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Attorney Docket No.	98,057-G
First Named Inventor	Jiandong Chen
Express Mail No.	EM442908971US
Total Pages	72

APPLICATION ELEMENTS	ACCOMPANYING APPLICATION PARTS
1. ☒ Transmittal Form with Fee	8. ☐ Assignment Papers
2. ☒ Specification (including claims and abstract, cover sheet) [Total Pages 72]	9. ☐ Power of Attorney
3. ☒ Drawings [Total Sheets 28]	10. ☐ English Translation Document (if applicable)
4. ☐ Oath or Declaration [Total Pages]	11. ☐ Information Disclosure Statement (IDS)
a. ☐ Newly executed	☐ PTO-1449 Form
b. ☐ Copy from prior application **[Note Boxes 5 and 17 below]**	☐ Copies of IDS Citations
i. ☐ Deletion of Inventor(s) Signed statement attached deleting inventor(s) named in the prior application	12. ☐ Preliminary Amendment
5. ☐ Incorporation by Reference: The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.	13. ☒ Return Receipt Postcard (Should be specifically itemized)
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c. ☐ Statement verifying above copies	16. ☒ Other: Certificate of Mailing by Express Mail; Sequence Listing

17. **If a CONTINUING APPLICATION**, check appropriate box and supply the requisite information:
☐ Continuation ☐ Divisional
☒ Continuation-in-part of prior application Serial No. 09/383,507.

APPLICATION FEES				
BASIC FEE				$690.00
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total Claims	29 -20=	9	x $18.00	$162.00
Independent Claims	3 - 3=		x $78.00	$.00
☐ Multiple Dependent Claims(s) if applicable			+$270.00	$.00
			Total of above calculations =	$852.00
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UTILITY PATENT APPLICATION TRANSMITTAL | Attorney Docket No. 98,057-G

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UTILITY (Rev. 11/18/97)

CERTIFICATE OF MAILING BY "EXPRESS MAIL"
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(Attorney Docket No.: 98,057-G)

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By: _____

Patent Application of: Jiandong Chen, Sudhir Agrawal, Ruiwen Zhang

Title: MDM2-Specific Antisense Oligonucleotides

X	Utility Patent Application Transmittal (2 Pages)
X	Patent Specification (72 pages, including cover sheet, claims, and Abstract)
X	Check ($852.00)
X	Postcard
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APPLICATION FOR UNITED STATES LETTERS PATENT

UNITED STATES PATENT AND TRADEMARK OFFICE

(Case No. 98,057-G)

Title: Mdm2-Specific Antisense Oligonucleotides

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MDM2-SPECIFIC ANTISENSE OLIGONUCLEOTIDES

BACKGROUND OF THE INVENTION

This is a continuation-in-part of U.S. Application Serial No. 09/383,507, filed August 26, 1999, which is a divisional of U.S. Application Serial No. 09/073,567, filed May 6, 1998, which is a continuation-in-part of U.S. Application Serial No. 08/916,834, filed August 22, 1997.

Field of the Invention

The invention relates to modulation of gene expression. In particular, the invention relates to modulation of gene expression through an antisense approach.

Summary of the Related Art

Regulation of gene expression is a complex process, and many aspects of this process remain to be understood. Aberrant gene expression appears to be responsible for a wide variety of inherited genetic disorders, and has also been implicated in numerous disease states including pathological conditions stemming from tumorigenic growth. A great deal of cancer related research pertains to the elucidation of the roles and interaction of tumor suppressor genes and oncogenes. For instance, tumor growth and malignancy are known to result from the combination of 1) activation of proto-oncogenes that promote cell growth with 2) inactivation of tumor suppressor genes that inhibit cell growth through cell growth arrest and induction of programmed cell death or apoptosis.

Several tumor suppressors have been identified. For instance, the identification and isolation of the WT1 gene is taught in Marshall et al., *Cell* **64**, 313-326 (1991). Coopers et al., *Cancer Invest.* **12**(1), 57-65 (1994) discloses that the WT1 gene product is a protein with four zinc fingers suspected to be a transcription factor. Further, Anderson and Spandidos Onco-Suppresso (1990) disclose the NF1 gene, another tumor suppressor, involved in the development

of neurofibromatosis functioning as a GTPase-activating protein for the GTP-binding protein $p21^{ras}$. In addition, Sager et al. *Science* **246**, 1406-1412 (1989) discloses several genes involved in the development of colon cancer, namely DCC, MCC and APC (FAP), suggesting that their products might also perform tumor suppressor functions.

To date however, the best characterized tumor suppressors are the RB and the p53 gene products (See, for example, "The p53 Pathway", Prives et al *J. Pathol.* **187**, 112-126 (1999)). Levine *Bioessays* **12**(2), 60-66 (1990) teaches RB gene inactivation in retinoblastoma. Notably, Levine et al. *Nature* **351**, 453-456 (1991), Weinberg et al. *Neur.* **11**, 191-196 (1991), and Williams et al. *Nature Genet.* **7**, 480-484 (1994), teach RB gene inactivation in many other tumor types including breast tumors, bladder carcinoma, and lung tumors.

Levine et al. *Nature* **351**, 453-456 (1991) teaches the p53 tumor suppressor gene's ability to encode a phosphoprotein suspected to play a pivotal role in fundamental biological processes in cell proliferation and differentiation. Lane *Br. Med. Bull.* **50**, (3)582-599 (1994) also teaches the p53 gene involvement in various types of tumors. In addition, Lowe et al. *Cell* **74**, 957-967 (1993) discloses that p53 is required to trigger apoptosis in response to chemotherapy and that p53 activation is an important factor in mediating the cytotoxic effects of many cancer treatments, including chemotherapy and radiation. *See also* Lowe et al. *Science* **266**, 807-810 (1994); Kastan et al. *Cancer Res.* **51**, 6304-6311 (1991); and Fritsche et al. *Oncogene* **8**, 307-318 (1993).

Further elucidation of the role of both RB and p53 regulation has led to the discovery that the mouse double-minute, or MDM2 oncogene is a negative regulator of wild-type p53 (Fakharzadeh et al., *EMBO J.* **10**:1565-1569 (1991); Piette et al., *Oncogene* **15**:1001-1010 (1997)). The human cDNA sequence (SEQ ID NO: 1) is disclosed in Volgelstein and Kinzler,

U.S. Patent No. 5,411860 and the mouse cDNA sequence (SEQ ID NO: 12) can be found in GenBank, Accession No. U40145. Cahill-Snyder et al., *Somatic Cell. Mol. Genet.* **13**:235-244 (1987) teach the identification of this oncogene because of its overexpression in a spontaneously transformed tumor cell line.

The MDM2-p53 autoregulatory feedback loop regulates the intracellular p53 function: the MDM2 gene is a target for direct transcriptional activation p53 and MDM2 protein is a negative regulator of p53. In addtition, MDM2 protein interacts with other cellular proteins that are involved in cell cycle regulation, including pRB, E2F1/DP1, p300 and p19ARF.

Overexpression of MDM2 is demonstrated in a variety of human tumors and may be due to one or more of three mechanisms: 1) gene amplification; 2) increased transcription; and/or 3) enhanced translation. Several studies have shown that overexpression of MDM2 is associated with poor prognosis in many human malignancies. Therefore, MDM2 plays a crucial role in cell cycle control and tumor transformation and growth.

The significance of MDM2 in cell regulatory functions has been extended to other interactions as well. Marechal et al., *Mol. Cell. Biol.* **14**:7417-7429 (1994) teaches the binding of the MDM2 protein to the ribosomal protein L5-5S RNA complex while Elenbaas et al., *Mol. Med.* **2**:(4)439-451 (1996) teaches MDM2 interaction with specific RNA structures.

Gastrointestinal cancers remain a major public health problem both in the USA and worldwide. In the United States, colorectal cancer is the second most common cancer in women and the third most common cancer in men. Although there has been considerable progress in research on the etiology, prevention, and experimental therapy of gastrointestinal cancers, no fully effective approaches are available currently for the treatment and prevention of this disease. MDM2 overexpression has been observed in human colorectal cancer.

MDM2 has been suggested as a novel target for cancer therapy, especially the p53-mdm2 interaction. The rationale for this at least includes the following: 1) MDM2 amplification and overexpression occur in many types of human cancers and the MDM2 levels correlate with poor prognosis in some cancers; 2) p53, which is negatively regulated by MDM2, plays a major role in tumor growth; 3) p53-mediated growth arrest and/or apoptosis have been suggested to be major mechanisms for currently used cancer therapy such as DNA damaging chemotherapeutics and radiation therapy; 4) loss of p53 function and/or overexpression of MDM2 is believed to correlate with tumor resistance to conventional therapy; and 5) MDM2 has displayed both p53-dependent and p53-independent activities in connection with its tumorigenic property. In the past few years, several strategies have been used to test the hypothesis that, by disrupting p53-MDM2 interaction, the negative regulation of p53 by MDM2 is diminished and the cellular functional p53 level will be increased, particularly following DNA damaging treatment, resulting in tumor growth arrest and/or apoptosis that leads to better therapeutic response. These approaches include the use of polypeptide, antibody, and antisense oligonucleotides.

Recently, we have successfully identified an anti-MDM2 antisense PS-oligo that effectively inhibits MDM2 expression in tumor cells containing MDM2 gene amplifications (Chen L. et al., *Proc Natl Acad Sci USA*, **95**: 195-200, 1998). Effective anti-human-MDM2 antisense PS-oligos were initially screened in two cell lines, JAR (choriocarcinoma) and SJSA (osteosarcoma), that contain wild type p53, amplified MDM2 genes, and overexpression of MDM2 oncoprotein. Of nine PS-oligonucleotides screened, Oligo AS5 (5'GATCACTCCCACCTTCAAGG-3'), which can hybridize to a position ~360 bp downstream of the translation start codon, was found to reproducibly decrease MDM2 protein levels in both cell lines by 3-5 fold at concentrations of 100-400 nM in the presence of Lipofectin. The

mismatched control Oligo M4 (5'-GATGACTCACACCATCAAGG-3') had no effect on MDM2 expression. Oligo AS5 was also shown to induce RNase H cleavage of the target MDM2 mRNA, resulting in truncation and degradation of the target. Further studies demonstrated that, following AS5 treatment, the p53 protein level was elevated and its activity was increased. A dose-dependent induction of p2l expression by AS5 was observed up to 6.6 fold at the optimal concentration of 200 nM, suggesting that p53 transcriptional activity be increased following inhibition of MDM2 expression. JAR cells treated with AS5 showed a significant increase in the levels of apoptosis. AS5 did not cause visible apoptosis in the H1299 cells that lack p53. These results suggested that apoptosis induced by AS5 be due to activation of p53 following MDM2 inhibition by the oligonucleotide.

In general, human cancer cell lines or tumor tissues with MDM2 gene amplifications or overexpression often have wild-type p53, presumably inactivated by MDM2. Several studies have now shown that overexpression of MDM2 is associated with poor prognosis in human malignancies, including osteosarcoma, soft tissue sarcoma, breast cancer, ovarian cancer, cervical cancer, oral squamous cell carcinoma, brain tumor, esophageal cancer, colorectal carcinoma, bladder cancer, urithelial carcinoma, leukemia, and large B cell lymphoma. These studies suggest that overexpression of MDM2 is associated with inactivation of wild-type p53, the inhibition of MDM2 expression in these tumors may lead to re-activation of p53 and induction of cell growth arrest of apoptosis of human tumors. It has been demonstrated that many cancer therapeutic agents exert their cytotoxic effects through activation of wild-type p53, and the restoration of wild-type p53 can increase the sensitivity of tumors to DNA-damaging agents. Restoration of wild-type p53 may also overcome the drug resistance of human cancers associated with dysfunction of p53. However, the activation of p53 by DNA damage such as

cancer chemotherapy and radiation treatment may be limited in cancers with MDM2 expression, especially those with MDM2 overexpression. Therefore, the inactivation of the MDM2 negative feed-back loop may increase the magnitude of p53 activation following DNA damage, thus enhancing the therapeutic effectiveness of DNA damaging drugs.

In addition to its interaction with p53, MDM2 has also been shown to bind to and interact with other cellular proteins such as the pRB, E2Fl, p300, ARF, p73, Numb and ribosomal protein, and RNA. Also, MDM2 has been shown to regulate the MyoD transcription factor. The biological consequences of these activities are not fully understood, but may be associated with transforming properties of MDM2 that may be p53-independent.

p53-Independent activity of MDM2 has been suggested by several reports and reviews. MDM2 gene products include several forms of polypeptide, representing alternatively spliced MDM2 variants. Various alternatively spliced MDM2 polypeptides have been found in several human tumors. Of the five forms of MDM2 analogs, only one retains p53 binding capability. However, cDNAs coding for all five forms of alternatively spliced MDM2 could independently transform NIH3T3 cells, indicating that these MDM2 transcripts have the p53-independent transforming ability. The effects of MDM2 overexpression on mammary tumorigenicity are seen in p53-null mice, indicating that MDM2 can cause transformation and tumor formation via a p53-independent mechanism. More recently, overexpression of MDM2 is shown to be associated with resistance to the antiproliferative effects of transforming growth factor β (TGF-β), which is p53-independent.

One of the advantages of the use antisense oligonucleotides or MDM2 specific antibody is that these agents may exert their effects in all MDM2 expressing tumors regardless of p53 status. This is very important since the p53-independent activity of MDM2 may play a role in

MDM2 tumorigenicity. Inhibition of MDM2 expression will ultimately prevent the interaction of MDM2 and other cellular protein. For example, the recent development of certain antisense anti-MDM2 oligonucleotide (Chen L. et al., *Proc Natl Acad Sci USA*, **95**: 195-200, 1998) has been shown to increase E2F-1 levels following microinjection (Blattner C. et al., *Mol. Cell. Biol.* **19**: 3704-3713, 1999).

One potential drawback is that these agents may have similar effects on normal host tissues, resulting in activation of endogenous p53 in normal tissues. The tolerance of increased p53 levels in normal tissues will be the key for the success of approaches aiming at elimination of MDM2 from the cells. The activation of p53 in normal tissues following DNA damaging treatment and resultant cell growth arrest and apoptosis are believed to be associated with side toxicities of conventional therapy. A recent study demonstrates that inhibition of p53 function can in fact prevent host toxicity associated with DNA damage treatment.

From the available literature, it is clear that efforts should be directed to identify modulators and potentiators of tumor suppressor genes expression as a possible therapeutic approach to tumorigenesis. The identification of regulatory proteins acting on tumor suppressors could potentially lead to the development of therapeutic approaches to tumorigenesis by the activation of tumor suppressor functions. Thus, there is a need for the identification of tumor suppressor regulators and of methods to activate tumor suppressors in the context of chemotherapy. In this context, there is a need to elucidate the mechanism(s) involved in the development of resistance to chemotherapy in tumor cells. There is therefore, a need to develop better tools to carry out such investigations. Ideally, such tools should take the form of improved antisense oligonucleotides that inhibit MDM2. Kondo et al., Oncogene 10:(10)2001-2006 (1995) has disclosed that antisense oligonucleotide phosphodiesters directed against MDM2

increase the susceptibility of tumor cells to cisplatin-induced apoptosis. Kondo et al. have recently disclosed that MDM2 gene induced the expression of the multidrug resistance gene (mdr1) and that of its product P-glycoprotein (P-gp) conferring resistance to the apoptopic cell death induced by DNA-damage inducing drugs. Kondo et al., Br. J. Cancer 74:(8)1263-1268 (1996) teach the antisense inhibition of the MDM2 gene to inhibit expression of p-gp in MDM2 expressing glioblastoma cells further suggesting that the MDM2 gene may play an important role in the development of MDR phenotype in human tumors. Unfortunately the oligonucleotides disclosed are phosphodiester oligonucleotides and thus not suitable as investigative tools for the purposes discussed herein, and as potential therapeutics for the treatment of neoplastic diseases. Therefore, there remains a need for improved antisense oligonucleotides. Such improved antisense oligonucleotides should preferably also represent potential therapeutics for the treatment of neoplastic disease.

SUMMARY OF THE INVENTION

The invention relates to modulation of gene expression. In particular, the invention relates to modulation of gene expression through an antisense approach. The invention provides better tools to identify modulators and potentiators of tumor suppressor gene expression as a possible therapeutic approach to tumorigenesis, and to elucidate the mechanism(s) involved in the development of resistance to chemotherapy in tumor cells. In particular the invention provides improved antisense oligonucleotides complementary to a portion of the MDM2-encoding RNA and methods for using such antisense oligonucleotides as analytical and diagnostic tools, as potentiators of transgenic animal studies and for gene therapy approaches, and as therapeutic agents. The invention further provides methods to activate tumor suppressors.

In addition, the invention also provides methods to augment and synergistically activate tumor suppressors in conjunction with the use of a DNA-damage inducing agent.

In a first aspect, the invention provides improved antisense oligonucleotides that inhibit the expression of the MDM2 protein. Such antisense oligonucleotides are complementary to a portion of MDM2-encoding RNA. Preferably, such antisense oligonucleotides contain one or a plurality of internucleoside linkages and optionally contain either deoxyribonucleosides, ribonucleosides, 2′-O-substituted ribonucleosides (preferably 2′-O-methyl ribonucleotides), or any combination thereof. Particularly preferred antisense oligonucleotides according to this aspect of the invention include chimeric oligonucleotides and hybrid oligonucleotides.

In a second aspect, the invention provides methods for activating a tumor suppressor in a cell, including providing to a cell expressing the MDM2 gene an antisense oligonucleotide according to the invention. In a preferred embodiment of this aspect, the invention provides a method for activating p53 tumor suppressor in a cell. In a particularly preferred embodiment, the present invention provides a method for synergistically enhancing DNA-damage induced activation of p53 in tumor cells by contacting tumor cells with both a DNA-damage inducing agent and an antisense oligonucleotide according to the invention.

In a third aspect, the invention provides a method for inhibiting tumor growth in a mammal, including a human, comprising administering to the mammal, which has at least one MDM2-expressing tumor cell present in its body, a therapeutically effective amount of an antisense oligonucleotide according to the invention for a therapeutically effective period of time. In a preferred embodiment of this aspect, the method comprises co-administration of a DNA-damage inducing agent.

In a fourth aspect, the invention provides a method for investigating the role of the MDM2 oncoprotein in tumorigenic growth. In the method according to this aspect of the invention, the cell type of interest is contacted with an antisense oligonucleotide according to the invention, resulting in inhibition of expression of the MDM2 oncogene in the cell. The antisense oligonucleotides can be administered at different points in the cell cycle, or in conjunction with promoters or inhibitors of cell growth to determine the role of the MDM2 protein in the growth of the tumor of interest.

In a fifth aspect, the invention provides *in vitro* and *in vivo* models to evaluate the therapeutic effectiveness of a recently identified anti-human-MDM2 antisense oligonucleotide (Chen L et al., *Mol Med* **5**: 21-34, 1999; Wang H. et al., Int *J. Oncol.* **15**: 653-660, 1999) in the treatment of human colorectal cancers when administered alone or in combination with conventional chemotherapeutic agents. Specifically, the primary goals are: 1) to obtain new oligos with better *in vivo* stability that can be used in future *in vivo* studies; 2) to determine the effects of anti-MDM2 oligos on human tumor cells with varying status of p53 and/or MDM2 expression; and 3) to identify the candidate cell lines that can be used in future *in vivo* studies. PS-oligonucleotide AS5-2 (5'TGACACCTGTTCTCACTCAC-3') was shown to have the highest activity in tested cell lines and was used in further studies. Thus far, 26 cell lines (16 types of human cancers) have been tested with AS5-2 in comparison with control oligonucleotides. Oligo AS5-2 significantly activated p53 activity in all cells with low levels of wild type p53, even in those with very a low level of mdm2 expression (Chen L. et al., *Mol Med* **5**: 21-34, 1999). AS5-2 has no effect on p53 levels in cells with null p53, H1299 and SK-N-MC, or those with mutant p53. Based on the above screening, a modified analog of AS5-2 with advanced antisense chemistry, Oligo AS, was designed and evaluated in subsequent studies. In cell lines that contain

wild type p53 and amplified MDM2 gene, SJSA and JAR, Oligo AS specifically inhibits MDM2 expression and p53 levels are elevated accordingly (Wang H. et al., *Int J. Oncol.* **15**: 653-660, 1999).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A, shows the nucleotide sequence of the MDM2 human cDNA comprising the nucleotide acid sequences set forth in the Sequence Listings as SEQ ID NO:1; Figure 1B, shows the nucleotide sequence of the murine MDM2 mRNA comprising the nucleotide acid sequences set forth in the Sequence Listings as SEQ ID NO:12.

Figure 2A is a representation of a Western blot showing the quantitation of MDM2 protein in cells treated with an antisense oligonucleotide according to the present invention; panel B is a representation of a Northern blot showing the quantitation of MDM2 mRNA in cells treated with an oligonucleotide according to the present invention; panel C is a representation of a Western blot showing the quantitation of p21/WAF protein in cells treated with an oligonucleotide of the invention.

Figures 3A-C are graphic representations showing the activation of the p53-responsive luciferase reporter expression by representative, nonlimiting, synthetic antisense oligonucleotides according to the present invention.

Figure 4 is a representation of a Western blot showing the detection of total p53 protein (panel A), p53-bound MDM2 protein complex (panel B), and total MDM2 protein (panel C).

Figure 5 is a reproduction of a photograph showing the morphology of cells treated with antisense oligonucleotides AS5 (panel A), and with control oligonucleotide M4 (panel B).

Figure 6 is a reproduction of a photograph of an Ethidium Bromide stained agarose gel showing the size shift of chromosomal DNA of cells treated with the antisense oligonucleotides of the invention.

Figure 7A and B are graphical representations showing the relative luciferase activity in JAR cells transfected with *BP100-luc* that had been treated with camptothecin (CPT) and oligonucleotide AS5. Figure 7B is a graphical representation showing the relative luciferase activity in MCF-7 cells incubated with CPT, BP100-luc and CMV-lacZ reporter plasmids, and oligonucleotides in the presence of cationic lipids.

Figure 8 displays the activation of p53 in JAR-BP100luc and MCF7-BP100luc cells by antisense oligonucleotides AS5-1 to AS5-7 and AS7-1 to AS7-5.

Figure 9A displays the sequence of the anti-MDM2 antisense phosphorothioate oligonucleotides. All sequences are displayed 3′ to 5′. The top sequence represents the non-coding strand of human MDM2. Fig. 9B displays the results of the screening of MDM2 antisense oligonucleotides. MCF-7 cells stably transfected with the BP100-luc reporter were treated with 50 nM of *MDM2* oligonucleotides for 20 hr. p53 transcriptional activity was determined as luciferase activity/unit protein. M4 is a 4 bp mismatch control of AS5. LC: control with lipofection alone. Fig. 9C displays the results of activation of p53 by HDMAS5-2, showing that it is sequence specific. JAR cells stably transfected with the BP100-luc reporter were treated with HDMAS5-2, mismatch control oligonucleotides of HDMAS5-2 (AS2M2: 2 bp mismatch. AS2M4: 4 bp mismatch) and an unrelated oligonucleotide K.

Figure 10A displays the induction of apoptosis by HDMAS5-2, which is shown to be p53-dependent. JAR cells stably transfected with an actin promoter-driven HPV E6 construct (JAR-E6) expressed no detectable p53 and significantly reduced level of MDM2 in Western blot analyses.

Identical amounts of total protein were loaded on each lane. Fig. 10B displays cells (treated and untreated) with oligonucleotide AS5-2 and demonstrates that JAR-E6 cells are resistant to apoptosis induction by AS5-2. JAR and JAR-E6 cells were treated with 200 nM of AS5-2 for 24 hr. HDMAS5-2 induced significant cell death in JAR cells, but not in JAR-E6 cells.

Figure 11 displays induction of p53 accumulation by oligonucleotide AS5-2 in different tumor cell lines. Cells were cultured on chamber slides, treated with 200 nM HDMAS5-2 or control oligonucleotide K for 20 hr, and stained for p53 expression using Pab1801. Treatment with oligonucleotide AS5-2 induced strong nuclear p53 accumulation in cells with low basal levels of wild type p53.

Figure 12A and B display autoradiograms demonstrating stabilization of p53 by inhibition of MDM2 expression. Fig. 12A shows that inhibition of MDM2 expression results in an increase of p53 level. Cells were treated with 200 nM of AS5-2 or control oligonucleotide K for 20 hr. p53 protein levels were detected by Western blot with antibody DO-1. Identical amounts of total protein were loaded on each lane. The double band in MCF-7 is due to a p53 polymorphic allele. Fig. 12B shows the results of the determination of p53 half life. SJSA cells were treated with 200 nM AS5-2 for 20 hr and the rate of p53 degradation was determined by a pulse-chase experiment. The half life of p53 was ~0.5 hr in untreated SJSA cells and >4 hr in AS5-2 treated cells as determined by desitometric analysis.

Figure 13A and 13B shows the results of induction of p53 transcriptional activation function. Cells stably transfected with the BP100-luciferase reporter were treated with antisense oligonucleotides at indicated concentrations for 20 hr. Luciferase activity/unit protein was determined and the magnitude of induction was shown compared to cells not treated with oligonucleotides. AS2M4: a 4 bp mismatch control of AS5-2. K: an unrelated oligonucleotide.

Figure 14 shows the induction of cell death by inhibition of MDM2 expression. Cells were treated with 200 nM AS5-2 or control oligonucleotide K for 24 hr and photographed. Examples of cell lines undergoing significant cell death characteristic of apoptosis are shown.

Figure 15A-D shows the inhibition of DNA synthesis by MDM2 antisense oligonucleotides. Cells were treated with 100 nM of oligonucleotides for 20 hr and labeled for 2 hr with BrdU. Incorporation rate of BrdU was determined by an ELISA assay and normalized to the number of viable cells. H1299 and 10(1) cells are human and mouse cells devoid of p53. LC: lipofectin treatment alone.

Figure 16 displays anti-tumor activities of anti-msm2 oligos administered alone or in combination with topoisomerase I inhibitor10-hydroxycamptothecin (HCPT). Animals bearing SJSA xenografts(average 150 mg) were treated with drugs by ip injection, at designated daily doses, 5 dose/week. Control: saline; AS5-2HM (mismatch control oligonucleotide); AS5-2H: anti-MDM2 hybrid oligonucleotide designed according the sequence of AS5-2; HCPT: a topoisomerase I inhibitor that induces DNA breaks. The numbers in parenthesis are daily doses (mg/kg/day).

Figure 17 displays representative tumor sizes of mice treated with antisense oligonucleotides according to the invention (alone or in combination with topoisomerase I inhibitor 10-hydroxycamptothecin (HCPT). Animals bearing SJSA xenografts (average 150 mg) were treated with drugs by ip injection at the designated daily dose, 5 dose/week. Oligo 1 is AS5-2HM, oligo 2 is anti-MDM2 oligo AS5-2H.

Figure 18 displays the results of anti-tumor activities of anti-MDM2 oligonucleotides adminstered alone or in combination with topoisomerase I inhibitor HCPT to animals bearing JAR xenografts (average 2,000 mg). Administration was by direct injection into the tupors at the

designated daily dose, 5 doses/week. Anti-MDM2 oligonucleotide: AS5-2H (5 mg/kg/day, 5 injections); anti-MDM2 oligonucleotide (5 mg/kg) + HCPT (3 mg/kg); control (saline); HCPT: 3 mg/kg/day.

Figure 19 displays the effects of anti-MDM2 antisense oligonucleotides on MDM2, p53 and p2l protein levels in LS174T cells in culture. Cells were incubated with Oligos AS at various concentrations for 24 hr, in the presence of Lipofectin (7 jig/ml). Identical total protein (20 tig) was analyzed by SDS-PAGE, followed by Western blotting.

Figure 20 displays the synergistic effects on MDM2, p53 and p2l protein levels of combination treatment of Anti-MDM2 oligo AS and the cancer chemotherapeutic agents in LS174T cells in culture.

Figure 21 displays the *in vivo* synergistic effects between anti-MDM2 oligonucleotide, Oligo AS and chemotherapeutic agents HCPT and 5-FU in mice bearing human colon cancer LS174T xenografts. Doses used in drugs alone or in combinations are: Oligo As and ASM, 20 mg/kg/day; HCPT, 3 mg/kg/day for the first week and 3 mg/kg every other day for remaining treatment period; and 5-FU, 10 mg/kg/day.

Figure 22 displays the representative animals and the removed tumor tissues following treatment with anti-MDM2 oligonucleotide, Oligo AS, alone or in combination with chemotherapeutic agents HCPT and 5-FU in mice bearing human colon cancer LS174T xenografts.

Figure 23 shows the effects of anti-MDM2 antisense oligonucleotides on MDM2, p53 and p2l protein levels in DLD-1 cells in culture. Cells were incubated with Oligos AS at various concentrations for 24 hr, in the presence of Lipofectin (7 gg/ml). Identical total protein (20 gg) was analyzed by SDS-PAGE, followed by Western blotting.

Figure 24 depicts the effects on MDM2, p53 and p2l protein levels of combination treatment of anti-MDM2 Oligo AS and the cancer chemotherapeutic agents in DLD-1 cells in culture. Cells were incubated with 200 nM Oligo AS or ASM in the presence of Lipofectin for 24 hr, followed by addition of various concentrations of chemotherapeutic agents HCPT (panel 1), adriamycin (panel II) and 5-FU (panel III) and incubation for additional 24 hr. At various concentrations. the effects on MDM2, p53 and p2l levels were evaluated following treatment with cytotoxic agents alone (lane A's) or pre-treatment with Oligo AS (lane B's) or Oligo ASM (lane C's).

Figure 25 displays the *in vivo* antitumor activities of Oligo AS administered alone or in combination with chemotherapeutic agents HCPT and 5-Fu in mice bearing human colon cancer DLD-1 xenografts. Doses used in drugs alone are 1, 10, and 20 mg/kg/day. Doses in combination therapy are the same as that with LS174T model.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to modulation of gene expression. In particular, the invention relates to modulation of gene expression of oncogenes. More specifically, the invention relates to the modulation of tumor suppressor activity. The invention provides methods to activate tumor suppressors. The invention provides improved antisense oligonucleotides complementary to a portion of the MDM2-encoding RNA and methods for using such antisense oligonucleotides as analytical and diagnostic tools, as potentiators of transgenic animal studies and for gene therapy approaches, and as potential therapeutic agents. The invention further provides methods to activate tumor suppressors. The invention also provides methods to augment and synergistically activate a tumor suppressor in conjunction with the use of a DNA-damage inducing agent. The invention still further provides *in vitro* and *in vivo* models to evaluate the

therapeutic effectiveness of a recently identified anti-human-MDM2 antisense oligonucleotide (Chen L et al., *Mol Med* **5**: 21-34, 1999; Wang H. et al., Int *J. Oncol.* **15**: 653-660, 1999) in the treatment of human colorectal cancers when administered alone or in combination with conventional chemotherapeutic agents. The patents and publications identified in this specification are within the knowledge of those skilled in this field and are hereby incorporated by reference in their entirety.

In a first aspect, the invention provides improved antisense oligonucleotides that inhibit the expression of the MDM2 gene. Such antisense oligonucleotides are preferably complementary to a portion of MDM2-encoding RNA shown in Figure 1 (SEQ ID NO:1). Preferably, such antisense oligonucleotides contain one or more modified internucleoside linkages and may optionally contain either deoxyribonucleosides, ribonucleosides or 2′-O-substituted ribonucleosides, or any combination thereof. Particularly preferred antisense oligonucleotides according to this aspect of the invention include chimeric oligonucleotides and hybrid oligonucleotides.

For purposes of the invention, "complementary" means being sufficiently complementary to have the ability to hybridize to a genomic region, a gene, or an RNA transcript thereof under physiological conditions. Such hybridization is ordinarily the result of base-specific hydrogen bonding between complementary strands, preferably to form Watson-Crick or Hoogsteen base pairs, although other modes of hydrogen bonding, as well as base stacking can also lead to hybridization. As a practical matter, such complementarity can be inferred for example from the observation of specific MDM2 expression inhibition.

Particularly preferred improved oligonucleotides according to the invention are complementary to all or a portion of a region of MDM2-encoding RNA that consists of a

nucleotide sequence selected from the group of sequences in the Sequence Listing as SEQ ID NOS:2-4, 7-11, and 13-24 (see Table 1, which also displays the corresponding antisense sequences). Preferably such improved oligonucleotides are complementary to a sequence that overlaps one of such sequences by at least one nucleotide. Preferably such improved antisense oligonucleotides according to this embodiment of the invention have nucleotide sequences of from about 12 to about 50 nucleotides. Most preferably, oligonucleotides have nucleotide sequences of from about 13 to about 19 nucleotides.

Table 1

NAME*	SEQ. ID NO.	SEQUENCE	TARGET MAP-SITE†
S4	2	5′-TTG GCC AGT ATA TTA TGA CT-3′	481-500
AS4	27	5′-AGT CAT AAT ATA CTG GCC AA-3′	
S5	3	5′-CCT TGA AGG TGG GAG TGA TC-3′	695-714
AS5	28	5′-GAT CAC TCC CAC CTT CAA GG-3′	
S7	4	5′-TGG ATC AGG ATT CAG TTT CA-3′	1018-1037
AS7	29	5′-TGA AAC TGA ATC CTG ATC CA-3′	
S1	7	5′-ACC TCA CAG ATT CCA GCT TC-3′	357-376
AS1	30	5′-GAA GCT GGA ATC TGT GAG GT-3′	
S2	8	5′-CCA GCT TCG GAA CAA GAG AC-3′	369-388
AS2	31	5′-GTC TCT TGT TCC GAA GCT GG-3′	
S3	9	5′-TCT ACC TCA TCT AGA AGG AG-3′	780-799
AS3	32	5′-CTC CTT CTA GAT GAG GTA GA-3′	

NAME*	SEQ. ID NO.	SEQUENCE	TARGET MAP-SITE†
S6	10	5′-TCC TTA GCT GAC TAT TGG AA-3′	1203-1222
AS6	33	5′-TTC CAA TAG TCA GCT AAG GA-3′	
S8	11	5′-TCA TGC AAT GAA ATG AAT CC-3′	1230-1249
AS8	34	5′-GGA TTC ATT TCA TTG CAT GA-3′	
S5 - 1	13	5′-ACA TCT GTG AGT GAG AAC AG-3′	669-688
AS5 - 1	35	5′-CTG TTC TCA CTC ACA GAT GT-3′	
S5 – 2	14	5′-GTG AGT GAG AAC AGG TGT CA-3′	675-694
AS5-2	36	5′-TGA CAC CTG TTC TCA CTC AC-3′	
S5 - 3	15	5′-TGA GAA CAG GTG TCA CCT TG-3′	680-699
AS5 - 3	37	5′-CAA GGT GAC ACC TGT TCT CA-3′	
S5 - 4	16	5′-ACA GGT GTC ACC TTG AAG GT-3′	685-704
AS5 - 4	38	5′-ACC TTC AAG GTG ACA CCT GT-3′	
S5 - 5	17	5′-TGG GAG TGA TCA AAA GGA CC-3′	704-723
AS5 - 5	39	5′-GGT CCT TTT GAT CAC TCC CA-3′	
S5 - 6	18	5′-GTG ATC AAA AGG ACC TTG TA-3′	709-728
AS5 - 6	40	5′-TAC AAG GTC CTT TTG ATC AC-3′	
S5 - 7	19	5′-AAG GAC CTT GTA CAA GAG CT-3′	717-736
AS5 - 7	41	5′-AGC TCT TGT ACA AGG TCC TT-3′	
S7 - 1	20	5′-TGA ACA TTC AGG TGA TTG GT-3′	998-1017
AS7 - 1	42	5′-ACC AAT CAC CTG AAT GTT CA-3′	

NAME*	SEQ. ID NO.	SEQUENCE	TARGET MAP-SITE†
S7 - 2	21	5′-ATT CAG GTG ATT GGT TGG AT-3′	1003-1022
AS7 - 2	43	5′-ATC CAA CCA ATC ACC TGA AT-3′	
S7 - 3	22	5′-AGG TGA TTG GTT GGA TCA GGA-3′	1007-1027
AS7 - 3	44	5′-TCC TGA TCC AAC CAA TCA CCT-3′	
S7 - 4	23	5′-ATT CAG TTT CAG ATC AGT TT-3′	1027-1046
AS7 - 4	45	5′-AAA CTG ATC TGA AAC TGA AT-3′	
S7 - 5	24	5′-GAT CAG TTT AGT GTA GAA TT-3′	1038-1057
AS7 - 5	46	5′-AAT TCT ACA CTA AAC TGA TC-3′	

*As used herein, sequences whose names begin with "S" are in the sense orientation, and sequences whose names begin with "AS" are in the antisense orientation. Furthermore, an "S" sequence and an "AS" sequence whose names have the same number designation are complementary in the Watson-Crick sense. For example, the sequence AS2 is complementary to S2:

5′-CCA GCT TCG GAA CAA GAG AC-3′ (SEQ ID NO: 8)

••• ••• ••• ••• ••• ••• ••

3′-GGT CGA AGC CTT GTT CTC TG-5′ (SEQ ID NO: 31)

† Numbering is according to SEQ ID NO: 1 in the Sequence Listing.

For purposes of the invention, the term "oligonucleotide" includes polymers of two or more deoxyribonucleosides, ribonucleosides, or 2′-substituted ribonucleoside residues, or any combination thereof. Preferably, such oligonucleotides have from about 8 to about 50 nucleoside residues, and most preferably from about 12 to about 30 nucleoside residues. The

nucleoside residues may be coupled to each other by any of the numerous known internucleoside linkages. Such internucleoside linkages include without limitation phosphorothioate, phosphorodithioate, alkylphosphonate, alkylphosphonothioate, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate, phospholinol, boranophosphate and sulfone internucleotide linkages.

In certain preferred embodiments, these internucleoside linkages may be phosphodiester, phosphotriester, phosphorothioate, or phosphoramidate linkages, or combinations thereof. The term oligonucleotide also encompasses such polymers having chemically modified bases or sugars and/ or having additional substituents, including without limitation lipophilic groups, intercalating agents, diamines and adamantane.

For purposes of the invention the term "2′-substituted" means substitution of the 2′ position of the pentose moiety with a C_1-C_6alkyl-O- (preferably methoxy) group, a C_1-C_6alkoxyC_1-C_6alkoxy (preferably $CH_3OCH_2CH_2O$-) group or with an -O-aryl or allyl group having 2-6 carbon atoms, wherein such alkyl, aryl or allyl group may be unsubstituted or may be substituted, *e.g.,* with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl, or amino groups. Alternatively, 2′-substituted means substitution with a hydroxy group (to produce a ribonucleoside), an amino or a halo group, but not with a 2′-H group. A "2′-O-substituted" nucleotide is a 2′-substituted nucleotide in which the linkage of the 2′ substituent to the pentose moiety is through an oxygen (*e.g.*, methoxy and methoxyethoxy).

Particularly preferred antisense oligonucleotides according to this aspect of the invention include chimeric oligonucleotides and hybrid oligonucleotides.

For purposes of the invention, a "chimeric oligonucleotide" refers to an oligonucleotide having more than one type of internucleoside linkage. One preferred embodiment of such a chimeric oligonucleotide is a chimeric oligonucleotide comprising a phosphorothioate, phosphodiester or phosphorodithioate region, preferably comprising from about 2 to about 12 nucleotides, and an alkylphosphonate or alkylphosphonothioate region. Preferably, such chimeric oligonucleotides contain at least three consecutive internucleoside linkages selected from phosphodiester and phosphorothioate linkages, or combinations thereof. For example, U.S. Patent No. 5,149,797 describes traditional chimeric oligonucleotides having a phosphorothioate core region interposed between methylphosphonate or phosphoramidate flanking regions. U.S. Patent Application Ser. No. 08/516,454, filed on August 9, 1995 discloses "inverted" chimeric oligonucleotides comprising one or more nonionic oligonucleotide region (*e.g.* alkylphosphonate and/or phosphoramidate and/or phosphotriester internucleoside linkage) flanked by one or more region of oligonucleotide phosphorothioate.

For purposes of the invention, a "hybrid oligonucleotide" refers to an oligonucleotide having more than one type of nucleoside. One preferred embodiment of such a hybrid oligonucleotide comprises a ribonucleotide or 2′-O-substituted ribonucleotide region, preferably comprising from about 2 to about 12 2′-O-substituted nucleotides, and a deoxyribonucleotide region. Preferably, such a hybrid oligonucleotide will contain at least three consecutive deoxyribonucleosides and will also contain ribonucleosides, 2′-O-substituted ribonucleosides, or combinations thereof. Examples of such hybrid oligonucleotides are disclosed in U.S. Patents Nos. 5,652,355 and 5,652,356.

Improved antisense oligonucleotides according to the invention have improved ability to inhibit MDM2 expression relative to prior art oligonucleotides. The exact nucleotide sequence

and chemical structure of an antisense oligonucleotide according to the invention can be varied within the parameters described herein, so long as the oligonucleotide retains its improved ability to inhibit MDM2 expression. This is readily determined by testing whether the particular antisense oligonucleotide is active by determining steady state levels of MDM2 protein, by determining the amount of MDM2 co-precipitated with p53, by assaying p53-inducible gene expression, by assaying p53 transcriptional activity, by analyzing total genomic DNA size, or by observing cell morphologies characteristic of apoptosis, all of which are described in detail in this specification.

Antisense oligonucleotides according to the invention may conveniently be synthesized on a suitable solid support using well known chemical approaches, including H-phosphonate chemistry, phosphoramidite chemistry, or a combination of H-phosphonate chemistry and phosphoramidite chemistry (*i.e.*, H-phosphonate chemistry for some cycles and phosphoramidite chemistry for other cycles). Suitable solid supports include any of the standard solid supports used for solid phase oligonucleotide synthesis, such as controlled-pore glass (CPG). (See, *e.g.*, Pon (1993) Methods in Molec. Biol. 20:465).

Antisense oligonucleotides according to the invention are useful for a variety of purposes. For example, they can be used as "probes" of the physiological function of MDM2 by being used to inhibit the activity of MDM2 in an experimental cell culture or animal system and to evaluate the effect of inhibiting such MDM2 activity. This is accomplished by administering to a cell or an animal an antisense oligonucleotide according to the invention and observing any phenotypic effects. In this use, antisense oligonucleotides according to the invention are preferable to traditional "gene knockout" approaches because they are easier to use and can be used to inhibit MDM2 activity at selected stages of tumor development or differentiation. Thus, antisense

oligonucleotides according to the invention can serve as probes to test the role of MDM2 in various stages of tumorigenesis.

Finally, antisense oligonucleotides according to the invention are useful in therapeutic approaches to benign and malignant tumors and other human diseases involving altered patterns of gene expression.

Antisense oligonucleotides according to the invention are useful for benign and malignant tumors to inhibit MDM2 expression to reactivate or enhance tumor suppressors such as p53 in tumors, and to enhance the p53-stimulatory effect of DNA-damage. In addition, several types of tumors (*e.g.*, osteosarcomas, gliomas, and breast cancer) have been found to overexpress MDM2. Antisense inhibition of MDM2 in these tumors reactivates p53 and reduces other p53-independent oncogenic activities of MDM2. Furthermore, the antisense oligonucleotides according to the invention are useful in the treatment of tumors that contain wild-type p53 to augment the effects of DNA-damaging based therapies. The anti-tumor utility of antisense oligonucleotides according to the invention is described in detail in the following paragraphs.

The present invention is also useful in enhancing gene therapy involving the introduction of p53 into p53-mutant tumors by inhibiting the MDM2-negative feed back loop.

For therapeutic use, antisense oligonucleotides according to the invention may optionally be formulated with any of the well known pharmaceutically acceptable carriers or diluents. This formulation may further contain one or more MDM2 inhibitor(s) and/or one or more additional MDM2 antisense oligonucleotide(s), or it may contain any other pharmacologically active agent, as discussed elsewhere in this specification.

In a second aspect, the invention provides methods for activating a tumor suppressor in a cell including contacting an antisense oligonucleotide according to the invention to a portion of MDM2-encoding RNA. In a preferred embodiment of this aspect, the invention provides a method for activating p53 tumor suppressor in a cell. In a particularly preferred embodiment, the present invention provides a method for synergistically enhancing DNA damage-induced activation of p53 by contacting tumor cells with a DNA-damage inducing agent and an antisense oligonucleotide according to the invention.

The term "tumor suppressor" is used to denote a gene involved in normal control of cellular growth and division which when inhibited contributes to tumor development. Representative examples of tumor suppressor genes include the RB gene isolated from a region deleted in retinoblastoma cells, the WT1 gene isolated from 11p3, which is occasionally deleted in Wilms' tumor types, the NF1 gene involved in neurofibromatosis, and the p53 gene, which has been found to be associated with a wide variety of tumors.

The term "p53" is used to designate the gene that encodes the nuclear phosphoprotein p53, which is involved in the regulation of fundamental biological processes in cell proliferation and cell death. This protein is also responsible for mediating cytotoxicity of anticancer therapy, and has been shown to act as a tumor-suppressor protein.

As used herein, the designation "DNA-damage inducing agent" and "cancer chemotherapeutic agent" are used to denote antineoplastic compounds that are capable of interfering with DNA synthesis at any stage of the cell cycle. As a practical matter, such activity can be inferred by the observation of cell apoptosis. Examples of such agents include but are not limited to alkylating agents (*e.g.*, mechlorethamine, chlorambucil, cyclophosphamide, mephalan, or ifosfamide), S-phase specific antimetabolites (*e.g.*, folate antagonists, purine antagonists, or

cytarabine), plant alkaloids (*e.g.*, vinblastine, vincristine, or podophyllotoxins), antibiotics (*e.g.*, doxorubicin, bleomycin, or mitomycin), nitrosureas (*e.g.*, carmustine, or lomustine), inorganic ions (such as cisplatin). Etoposide and cisplatin are other chemotherapy drugs that are known to activate p53 by causing DNA damage and are contemplated for use in the invention.

The third aspect of the invention sets forth a method for inhibiting tumor growth in a mammal, including a human, comprising administering to the mammal, which has at least one tumor cell present in its body, a therapeutically effective amount of an antisense oligonucleotide according to the invention for a therapeutically effective period of time. In the method according to this aspect of the invention a therapeutically effective amount of an antisense oligonucleotide according to the invention is administered for a therapeutically effective period of time to an animal, including a human, which has at least one tumor cell present in its body.

As used herein the term "tumor growth" is used to refer to the growth of a tumor cell. A "tumor cell" is a neoplastic cell. A tumor cell may be benign, *i.e.* one that does not form metastases and does not invade and destroy adjacent normal tissue, or malignant, *i.e.* one that invades surrounding tissues, is capable of producing metastases, may recur after attempted removal, and is likely to cause death of the host.

The terms "therapeutically effective amount" and "therapeutically effective period of time" are used to denote known treatments at dosages and for periods of time effective to reduce tumor cell growth. Preferably, such administration should be parenteral, oral, sublingual, transdermal, topical, intranasal or intrarectal. When administered systemically, the therapeutic composition is preferably administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.01 μM to about 10 μM. For localized administration, much lower concentrations than this may be effective, and much higher concentrations may be tolerated.

Preferably, a total dosage of MDM2 inhibitor will range from about 0.1 mg oligonucleotide per patient per day to about 200 mg oligonucleotide per kg body weight per day.

In a preferred embodiment of this aspect, the method also includes the administration of a DNA-damage inducing agent. According to another embodiment, one or more of the oligonucleotides of the invention may be administered to an animal. This aspect of the invention provides methods for inhibiting tumor growth comprising administering to an animal, including a human, more than one antisense oligonucleotide according to the invention either sequentially or simultaneously in a therapeutically effective amount and for a therapeutically effective period of time.

In a fourth aspect, the invention provides a method for investigating the role of the MDM2 oncoprotein in cell development and differentiation and in tumorigenic growth of cells that are overexpressing MDM2. In the method according to this aspect of the invention, the cell type of interest is contacted with an antisense oligonucleotide according to the invention, resulting in inhibition of expression of the MDM2 oncogene in the cell. The antisense oligonucleotides can be administered at different points in the cell cycle, in conjunction with promoters or inhibitors of cell growth, or with DNA replication inhibitors to determine the role of the MDM2 protein in the growth of the tumor of interest.

In a fifth aspect, the invention provides *in vitro* and *in vivo* models to evaluate the therapeutic effectiveness of a recently identified anti-human-MDM2 antisense oligonucleotide (Chen L et al., *Mol Med* **5**: 21-34, 1999; Wang H. et al., Int *J. Oncol.* **15**: 653-660, 1999) in the treatment of human colorectal cancers when administered alone or in combination with conventional chemotherapeutic agents. Alternatively, this aspect of the invention provides a treatment of human colorectal cancers by administering such an anti-human-MDM2 antisense

oligonucleotide in combination with radiation therapy (using radiation therapy protocols as known in the art). The selected antisense mixed-backbone oligonucleotide was evaluated for its *in vitro and in vivo* antitumor activity in human colon cancer models: LS 174T cell line containing wild type p53 and DLD- I cell line containing mutant p53.

We demonstrate below that antisense inhibition of MDM2 expression activates p53 in tumor cells containing either low or high levels of MDM2. Importantly, inhibition of MDM2 expression in cells with low levels of p53 uniformly results in p53 accumulation and increase of p53 activity. This response occurred in nearly all of the tumor and non-transformed cells tested. The only exceptions were HPV-positive cells, which have an independent E6-mediated mechanism of p53 degradation. The accumulation of p53 is due to a prolonged half-live, therefore, MDM2 plays a general role in maintaining p53 at low levels through degradation.

Our observations suggest that MDM2 overexpression is not the only indicator for p53 being in a functionally suppressed state. In tumor cells with low levels of MDM2 (which usually correlate with low levels of wild-type p53), MDM2 is still an active inhibitor of p53 through degradation. It is thus possible that the levels of MDM2 and p53 change during tumor development, such as due to p53-independent induction of MDM2 expression. Shaulian, *Oncogene* **15**, 2717-2725 (1997). Thus MDM2 may be a causative factor in tumor development even when it is not overexpressed. Alternatively, these tumors may have successfully evaded the surveillance mechanism that signals for p53 activation, leaving the *MDM2* regulatory loop at a pre-malignant state which prevents p53 accumulation.

The ubiquitous role of MDM2 in regulating p53 turnover suggests that many signals (such as DNA damage, hypoxia, oncogene activation) that can lead to p53 stabilization may act through modulation of p53-MDM2 interaction or MDM2 function. It has been demonstrated that DNA

damage activation of DNA-PK phosphorylates p53 and MDM2 and inhibits MDM2 binding. Mayo *et al.*, *Cancer Res.* 57, 5013-5016 (1997); Shieh *et al.*, *Cell* **91**, 325-334 (1997). Whether other p53-inducing signals act through similar mechanisms remains to be tested.

Our observations suggest that MDM2 overexpression is not the only indicator for p53 being in a functionally suppressed state. In tumor cells with low levels of MDM2 (which usually correlate with low levels of wild-type p53), MDM2 is still an active inhibitor of p53 through degradation. It is thus possible that the levels of MDM2 and p53 change during tumor development, such as due to p53-independent induction of MDM2 expression. Shaulian, *Oncogene* **15**, 2717-2725 (1997). Thus, MDM2 may be a causative factor in tumor development even when it is not overexpressed.

Our observations also show *in vitro* antitumor activity was found in both cell lines (i.e. LS 174T cell line containing wild type p53 and DLD- I cell line containing mutant p53), resulting from specific inhibition of MDM2 expression. Following i.p. administration of the selected oligonucleotide, *in vivo* antitumor activity was observed in nude mice bearing LS 174T or DLD-I xenografts in a dose-dependent manner. More interestingly, *in vivo* synergistically or additive therapeutic effects of MDM2 inhibition and the cancer chemotherapeutic agents 10-hydroxycamptothecin and 5-fluorouracil were observed in both tumor models. These results suggest that MDM2 have a role in tumor growth through both p53-dependent and p53-independent mechanisms. We speculate that MDM2 inhibitors such as antisense anti-MDM2 oligonucleotides have a broad spectrum of antitumor activities in human cancers regardless of p53 status.

The results of this study suggest that MDM2 is a useful drug target in many tumor types, even when it is not a causative factor during tumor development. Many types of tumors with wide impact or high mortality rate, such as tumors of the breast, liver, prostate, and brain, have p53

mutation frequencies of 20-30%. Hollstein *et al.*, *Nucleic Acids Res.* **22**, 3551-3555 (1994). Results presented below (see Table 2 and related text) demonstrate that the AS5-2 oligonucleotide causes about 70-80% cell death in C33A cells, which have mutant p53 and high MDM2 levels, indicating that oligonucleotides according to the invention can have an effect in cells (and potentially tumors) without a functional p53. Therefore, inhibitors of MDM2 may be useful for the majority of such cases.

Furthermore, MDM2 may have p53-independent functions that contribute to tumor development, such as regulation of Rb and E2F/DP1 (Xiao *et al.*, *Nature* **375**, 694-698 (1995); and Martin *et al.*, *Nature* **375**, 691-694 (1995)), and possibly regulation of the p53 homolog p73. Kaghad *et al.*, *Cell* 90, 809-819 (1997)). Inhibition of MDM2 expression will abolish these functions as well.

With respect to the *in vitro* and *in vivo* models that demonstrate the role of MDM2 in human colon cancer, five points have been demonstrated. First, the novel anti-NMM2 mixed-backbone oligonucleotide, Oligo AS, specifically inhibits MDM2 expression in both LS174T and DLD- 1 cells, with dose-dependent inhibitory effects on cell growth, regardless of p53 status. Second, in a dose-dependent manner, the *in vivo* antitumor effects of Oligo AS were observed in LS174T and DLD-1 tumor models in nude mice. Third, *in vivo* synergistic or additive therapeutic effects were found in both LS174T and DLD-1 models after combination therapy with Oligo AS and conventional cancer chemotherapeutic agents HCPT and 5FU. Fourth, in LS 174T cells, combination treatment with Oligo AS and cancer chemotherapeutic agents HCFIT, adriamycin and 5-FU significantly elevated chemotherapeutic agent-induced p53 and p21 levels, resulting from inhibition of chemotherapeutic agent-induced MDM2 expression, indicating that the *in vivo* synergistic effects between Oligo AS and conventional chemotherapeutic agents be associated

with a p53-dependent pathway in cancers containing wild type p53 expression. Finally, in DLD-1 cells, combination treatment with Oligo AS and cancer chemotherapeutic agents HCPT, adriamycin and 5-FU had no effect on the mutant p53 levels. However, Oligo AS specifically inhibited the chemotherapeutic agent-induced MDM2 expression and increased p21 levels, indicating that the *in vivo* synergistic or additive effects between Oligo AS and conventional chemotherapeutic agents is independent to p53 but associated with MDM2 and possibly with p21.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature. Those skilled in the art will appreciate that modifications and variations for the following can be made without exceeding either the spirit or scope of the invention.

EXAMPLES

Example 1

Inhibition of MDM2 Expression By AS5

Choriocarcinoma JAR cells (ATCC) containing wild-type p53 were grown in DMEM medium supplemented with 1% FBS according to standard cell culture techniques. Cells were then treated for 18 hours with growth medium containing 50, 100, 200, and 500 nM of antisense oligonucleotides AS4 (SEQ ID NO:27), AS5 (SEQ ID NO:28), AS1 (SEQ ID NO:30), AS2 (SEQ ID NO:31), AS3 (SEQ ID NO:32), AS6 (SEQ ID NO:33), AS8 (SEQ ID NO:34), AS7 (SEQ ID NO:29) (complementary to sequences S4, S5, S1, S2, S3, S6, S8, and S7, respectively, of MDM2-encoding RNA), with 500 nM of a control oligonucleotide K (5′-CAGAGCCTTCATCTTCCCAG-3′; SEQ ID NO:6) complementary to an ion channel, or with 500 nM of a mismatch control oligonucleotide M4 (5′-GATGACTCACACCATCATGG-3′;

SEQ ID NO:5) containing four mismatches within the same portion of MDM2-encoding RNA, and 7 μg/ml Lipofectin (Gibco BRL).

Treated cells were then harvested and lysed in lysis buffer (50 mM Tris pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP40). Total protein was then extracted according to standard methods (see *e.g.*, Current Protocols in Molecular Biology, John Wiley & Sons Inc. (1995)). Two mgs of total protein were mixed with 100 μl of hybridoma supernatant containing an anti-MDM2 monoclonal antibody 2A10 (Chen et al., Mol. Cell. Biol. 13:4107-4114 (1993)), and with 20 μl of packed protein A-Sepharose beads (Sigma, St. Louis, MO). Immunoprecipitates were obtained by incubation of the mix at 4°C for 3-5 hours on a rotator. The beads were then washed with lysis buffer three times. Immunoprecipitates were then boiled in loading dye (0.3 M Tris-HCl pH 8.8, 0.2% SDS, 10% glycerol 28 mM 2-mercaptoethanol and 24 μg/ml brompohenol blue). Samples were fractionated by electrophoresis on an SDS polyacrylamide gel with a 5% stacking gel and a 10% separation gel. The gel was then transferred onto an Immobilon P membrane (Millipore, Bedford, MA). The membrane was then blocked with PBS/5% non-fat milk+1/500 polyclonal serum for 1 hr. The membrane was then washed with PBS/5% milk and I^{125} protein A (0.2 μCi/ml) for 1.5 hours. The filter was then washed with PBS and 0.1% Tween20 and exposed to a phosphorimaging screen.

As shown in Figure 2A, treatment with oligonucleotide AS5 resulted in approximately 3-5 fold inhibition of MDM2 expression at concentrations between 100 and 400 nM. This effect was not observed with an oligonucleotide targeted to an unrelated ion channel gene (oligonucleotide K) or an AS5 mismatch control oligonucleotide containing 4 base mismatches with the same target (oligonucleotide M4).

Example 2

Alteration of MDM2 RNA by AS5

JAR cells were treated with 200 nM of antisense oligonucleotide AS5 (SEQ ID NO:28) with no oligonucleotide, with 200 nM of control oligonucleotide M4 (SEQ ID NO:5). After 18 hours, the treated cells were harvested and RNA was purified and quantitated according to standard methods. (see *e.g.*, *Current Protocols in Molecular Biology*, John Wiley & Sons Inc. (1995)). Twenty μg of total RNA were run on a 1% agarose denaturing gel. MDM2 mRNA was detected by hybridization using an EcoRI-NcoI fragment specific for the human MDM2 cDNA between nucleotides 310-1633. The filter was then stripped and reprobed with a 1.2 kb fragment, which is a full length human GAPDH cDNA to normalize values on the basis of loading variations, according to standard methods.

As shown in Figure 2B, treatment with oligonucleotide AS5 (SEQ ID NO:28) resulted in a slight decrease in the molecular weight of the MDM2 mRNA band. This is consistent with RNase H cleavage at the site of the oligonucleotide AS5 hybridization (approximately 700 bp from the 5′ end), which would reduce the molecular weight of the mRNA (normally approximately 5500 nt long) by approximately 12%. Most important, as shown in Fig. 2B, treatment with the AS5 resulted in 2.5 fold increase of MDM2 mRNA consistent with p53 activation in response to decreased MDM2 protein levels following inhibition of MDM2 expression. Comparable results were also obtained using osteosarcoma SJSA cells (ATCC) (data not shown).

Example 3

Induction of p21/WAF1 Expression by Oligonucleotide AS5

To assess the ability of the oligonucleotides of the invention to induce the expression of a p53-inducible gene, p21/WAF1 levels were examined in oligonucleotide-treated JAR cells. Total protein was purified from control oligonucleotide-treated cells and from antisense oligonucleotide-treated cells (200nM oligonucleotide AS5) as described in Example 1. Equal amounts of purified total protein were immunoprecipitated and analyzed by Western blotting carried out for MDM2 detection using a polyclonal rabbit anti-human p21/WAF1 serum. Following hybridization, the blots were exposed to XAR film (Eastman Kodak, Rochester, NY) and the autoradiograms are quantitated by phosphoroimaging.

The results shown in Figure 2C demonstrate a dose dependent induction of p21/WAF1 up to 6.6 fold (corresponding to 200 nM oligonucleotide) in antisense oligonucleotide-treated cells, relative to controls treated cells (lanes designated as *No oligo*, *K* and, *M4*).

Example 4

Activation of a p53-Responsive Reporter Gene by AS5

To measure p53 transcriptional activity in response to treatment with the oligonucleotides of the invention, a p53 responsive luciferase reporter *BP100-luc,* containing the p53 binding site from intron I of the MDM2 gene (Wu et al., J. Gene. Dev. 7:1126-1132 (1993), was transfected into JAR cells with a neomycin-resistant plasmid *pCMV-neo-Bam* (Baker et al., Science 249:912-915 (1990)), according to conventional methods (See *e.g.*, *Molecular Cloning,* 2d Edition, Cold Spring Harbor Laboratory Press (1989)). Transfected cells were then plated and stable G418-resistant colonies were pooled and treated with no oligonucleotide, with 200 nM of control oligonucleotide M4 (SEQ ID NO:5), and with 200 nM of antisense oligonucleotide AS5

(SEQ ID NO:28). After 24 hours, luciferase activity levels in the oligonucleotide-treated cells were determined.

Figure 3 shows the results obtained from at least four experiments for each data point. As shown in Figure 3A, oligonucleotide AS5 activated the p53-responsive reporter expression by 7 fold. JAR cells stably transfected with a luciferase reporter driven by the thymidine kinase gene promoter (*JAR-TK-luc*) and H1299 cells containing no p53 that had been stably transfected with BP100-luciferase (*H1299-BP1000-luc*) were also tested. As shown in Figs. 3B and 3C, oligonucleotide AS5 did not activate the reporter gene in these control experiments. Similar results were also observed using AS5 in osteosarcoma SJSA cells (data not shown).

Example 5

Reduction of p53-MDM2 Complex by the Antisense Oligonucleotides

Protein lysates from JAR cells treated with antisense oligonucleotides as described in Example 1 were immunoprecipitated with anti-p53 monoclonal antibody Pab421 (Figs. 4A and 4B) (Harlow et al. J. Virol. 39:861-869 (1981)) or with polyclonal antibody 2A10 (panel C) according to the methods described in Example 1. The gel was then transferred onto an Immobilon P membrane (Millipore, Bedford, MA) to detect MDM2 co-precipitates.

As shown in Figure 4A, p53 levels did not change following treatment with oligonucleotide AS5. As shown in panel B, the amount of MDM2 co-precipitated with p53 were reduced by oligonucleotide AS5. The results demonstrated that a marked reduction in the MDM2-p53 complex is detected by Western blotting (see Figure 4B), strongly indicating that antisense oligonucleotide treatment activates p53 by increasing the levels of free p53 but not total p53.

Example 6

Induction of Apoptosis by Antisense Oligonucleotides

JAR cells (ATCC) were grown in DMEM medium supplemented with 1% FBS according to standard cell culture techniques. Cells were then treated for 30 hours with growth medium containing 400 nM of either antisense oligonucleotide AS5 (SEQ ID NO:28) complementary to a portion of MDM2-encoding RNA or control oligonucleotide M4 (SEQ ID NO:5), and 7 μg/ml lipofectin (Gibco BRL Paisley, UK). Treated cells were then photographed using a phase contrast microscope.

As shown in the Figure 5, oligonucleotide AS5 induced significant cell death. Dying cells show the morphology characteristic of apoptosis, such as membrane blebbing and shrinkage. Control oligonucleotide M4 (SEQ ID NO:5) induced significantly less apoptosis.

Example 7

Interchromosomal DNA Cleavage in Floating Cells

JAR cells were treated for 24 hours as described in Example 6. Floating cells were harvested and chromosomal DNA was extracted according to standard techniques (Liu et al., Cell 86:147-157 (1996)). Purified DNA was then analyzed by agarose gel electrophoresis on a 2% agarose gel. Following electrophoresis the gel was stained with 0.5 μg/ml ethidium bromide.

As shown in Figure 6, genomic DNA purified from oligonucleotide AS5 treated cells showed nucleosomal-sized low molecular weight bands characteristic of apoptosis. Treatment of H1299 cells, (which lack p53) did not cause visible apoptosis (data not shown). These results suggest that oligonucleotide AS5 induced apoptosis is attributable to the activation of p53.

Example 8

Co-activation of p53 by AS5 and DNA-Damage

JAR cells stably transfected with *BP100-luc* as described in Example 4 were treated with the Topoisomerase I inhibitor camptothecin (CPT) and with 100 nM and 200 nM of either antisense oligonucleotide AS5 (SEQ ID NO:28), control oligonucleotide M4 (SEQ ID NO:5), or Lipofectin (no oligonucleotide control) for 48 hours. Induction of p53 activity was measured by luciferase assay as described in Example 4.

The results are displayed in Figs. 7A-C. As shown in Figures 7A and B, CPT alone activated the p53 reporter only by 3-4 fold, incubation with 200 nM of oligonucleotide AS5 resulted in a 17-fold activation of the p53 reporter. Also as shown in Figures 7A and B, co-incubation with CPT and oligonucleotide AS5, however, resulted in up to 90 fold induction of p53 activity. A similar synergistic effect between AS5 and CPT was also observed in MCF-7 cells, a breast tumor cell line with wild-type p53 but no amplification of MDM2 (Figure 7C). These results demonstrate that inhibition of MDM2 can synergistically cooperate with the effect of DNA damage and induce p53 transcriptional activity to high levels.

Examples 9-14

Investigation of Oligonucleotides Targeted to Regions Around the AS5 and AS7 Targeted Regions

Antisense oligonucleotides targeted to AS5 (SEQ ID NO:28) and AS7 (SEQ ID NO:29) sequences within the MDM2 RNA were found to be the most effective in inducing p53 activity among the initial antisense oligonucleotides tested. This prompted us to further investigate antisense oligonucleotides targeted to the region around where the AS5 and AS7 oligonucleotides were targeted. Antisense oligonucleotides SEQ ID NOs: 35-46 were selected to

target (*i.e.*, be complementary to) sequences SEQ ID NOs: 13-24 within the human MDM2 mRNA, which target sequences overlapped or flanked the AS5 and AS7 target sequences. See Table 1, *supra.*

The following protocols were employed in each of the experiments disclosed in Examples 9-14, unless otherwise noted.

Synthesis of oligodeoxynucleotides. Phosphorothioate oligodeoxynucleotides were synthesized using β-cyanoethyl phosphoramidite chemistry on an automated synthesizer (Expedite 8909, Perceptive Biosystems, Framingham, MA) and purified by preparative reverse-phase high performance liquid chromatography. Twelve 20-mer phosphorothioate antisense oligodeoxynucleotides (AS5-1 to AS5-7 and AS7-1 to AS7-5) were synthesized for this screen (Figure 8). A 2 bp mismatch control antisense oligonucleotide (AS2M2: 5′-TGACACTTGTTCTTACTCAC-3′; SEQ ID NO: 25) and a 4 bp mismatch control antisense oligodeoxynucleotide (AS2M4: 5′-TGACTCTTGTCCTTACTCAC-3′; SEQ ID NO: 26) targeted to the S5-2 sequence of human MDM2 RNA were also synthesized. The oligodeoxynucleotide K (SEQ ID NO: 6) was a control against an unrelated target.

Cell lines. JAR, JEG-3, SJSA, MCF-7, U87-MG, SK-N-SH, U2OS, Caski, C33A, DLD-1, and A549 cells were obtained from the ATCC. WI-38, JeKin, HepG2, LS180, HT1080, G361, PA-1, and Lncap cells were obtained from the cell culture core lab of LSU Medical Center. H1299, MCF-7, MDA-MB-231, and Hela cells were from Dr. Arnold J. Levine (Princeton University). The 101 cell line was provided by Dr. James Gnarra (LSU Medical Center). The SLK cell line was kindly provided by Dr. Om Prakash (Ochsner Foundation). All cells were grown in DMEM with 10% fetal bovine serum (FBS).

Antisense oligonucleotide treatment. Cells were cultured in DMEM medium with 10% FBS. Cell lines normally grown in other types of medium were also adapted to growth in DMEM with 10% FBS before the treatment. Before addition of oligonucleotides, cells were refed with DMEM containing 1% FBS. Lipofectin (Gibco BRL) was incubated with serum-free DMEM medium for 45 min, then mixed with the oligonucleotides for 10 min and added to the cell culture. The final concentration of Lipofectin was 7 μg/ml, final concentration of FBS was 0.75%. Cells were incubated with oligonucleotides and Lipofectin for 18-24 hr as indicated.

Western blot. Cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% Na deoxycholate) and 100 μg of the protein lysate were fractionated by SDS-polyacrylamide gel electrophoresis (SDS PAGE) and transferred to Immobilon P filters (Millipore). The filters were blocked for 5 min with 5% non-fat dry milk, incubated with anti-p53 antibody DO-1 or an anti-human MDM2 rabbit serum, then incubated with protein A-peroxidase (Promega), washed, and developed using the ECL-plus reagent (Amersham). All incubations were carried out in phosphate-buffered saline with 5% non-fat milk and 0.1% Tween-20.

Stable transfection of cell lines. Cells were co-transfected with the BP100-luciferase reporter plasmid or pActin-E6 plasmid and a G418-resistant marker plasmid pCMV-neo-Bam using the calcium phosphate precipitation method. Transfected cells were grown in medium with 750 μg/ml G418 until colonies appeared. Individual colonies were isolated and expanded into cell lines. The JAR-BP100-luc is a clonal cell line isolated by diluting a pool of BP100-luciferase transfected JAR cells from a previous experiment. Chen *et al.*, *Proc. Natl. Acad. Sci. U. S. A.* **95**, 195-200 (1998).

Immunofluoresence staining. For p53 staining, cells were treated with oligonucleotides for 20 hr, fixed with acetone-methanol (1:1) for 3 min, then blocked with PBS+10% normal goat serum (NGS) for 20 min, and incubated with Pab1801 hybridoma supernatant for 2 hr. In order to stain for

MDM2, cells were fixed in 4% paraformaldehyde in PBS for 20 min, blocked with PBS+10% NGS for 20 min, and incubated with 2A9 hybridoma supernatant at 1/100 dilution in PBS+10% NGS for 2 hr. Slides were washed with PBS+0.1% Triton X-100, incubated with FITC-goat-anti-mouse IgG in PBS+10% NGS for 1 hr, washed with PBS+0.1% TritonX-100 and mounted.

Determination of p53 half life. SJSA cells were treated with 200 nM AS2 for 20 hr. Cells were incubated with DMEM (without methionine) with 2% dialyzed FBS, 50 uCi/ml 35S-EXPRESS (NEN) for 2 hr and refed with regular medium. Sample plates were collected at indicated time points and lysed with lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP40, 1 mM PMSF). Cell lysates with identical levels of radioactivity (~$2x10^7$ CPM) were immunoprecipitated with Pab421 and Pab1801, washed with 50% SNNTE buffer (25 mM Tris, pH 7.4, 2.5 mM EDTA, 2.5% sucrose, 1% NP-40, 250 mM NaCl), and fractionated by SDS PAGE. P53 was detected by autoradiography.

Determination of cell proliferation rate. Cells were treated with 100 nM of oligonucleotides for 20 hr, labeled with BrdU for 2 hr, and incubated with MTS reagent (Promega) for 1 hr. Relative cell viability was determined by measuring OD at 490 nm (reduction of MTS substrate by mitochondria activity). Cells were then fixed and the level of BrdU incorporation was determined using a chemiluminscence ELISA assay (Boehringer Mannheim). The rate of DNA synthesis was determined as BrdU incorporation/OD 490.

Example 9

Optimization of MDM2 Antisense Oligonucleotide

JAR cells or MCF7 cells were stably transfected with the p53-responsive BP100-luciferase reporter gene were incubated with 100 nM of oligonucleotides AS5-1 to AS5-7 and AS7-1 to AS7-5 in the presence of cationic lipids for 20 h. p53 transcriptional activation

function was determined by measuring luciferase activity. Oligonucleotides AS5 and AS7 were used as positive controls. A missense mutant of AS5M4 was used as a negative control.

The results shown in Fig. 8 demonstrate that all of the oligonucleotides tested were effective in induction of p53 activity in both tumor cell lines. The oligonucleotide AS5-2 is the most potent in activation of p53 and was further tested in animal tumor models, *infra.*

The p53 activation assays demonstrated that several of the secondary oligonucleotides were more effective than AS5 in causing activation of p53 (Figure 9B). The AS5-2 oligonucleotide was the most potent of this group and was chosen for further characterization. AS5-2 was five-fold more efficient than AS5 in MCF-7cells (Figure 9B) and two-fold more efficient than AS5 in JAR cells (not shown) at a concentration of 50 nM. Similar to the AS5 oligonucleotide, AS5-2 treatment also inhibited MDM2 protein expression (not shown). The effect of AS5-2 is sequence specific. Introduction of two or four nucleotide mismatches into the sequence significantly inhibited its ability to activate p53 (Figure 9C). When AS5-2 and AS5 (each 20 nucleotides) were shortened to 18 nucleotides from one or both ends, the ability to activate p53 was also significantly reduced (not shown).

Example 10

Apoptotic Function of AS5-2 is p53-dependent

Similar to the parent AS5 oligonucleotide, AS5-2 also induces apoptosis in JAR cells. In order to further delineate whether AS5-2 induces apoptosis through activation of p53, a JAR cell line expressing the E6 oncogene of HPV16 was created (JAR-E6). Expression of E6 under the actin promoter resulted in degradation of p53, as demonstrated by the loss of p53 protein in a Western blot (Figure 10). Interestingly, the level of MDM2 expression also decreases significantly in JAR-

E6 cells, suggesting that in addition to gene amplification, activation by p53 is an important mechanism of MDM2 overexpression in this cell line.

When treated with 200 nM of AS5-2, which efficiently induced apoptosis in parental JAR cells, JAR-E6 cells showed little apoptosis (Figure 10). This result suggests that AS5-2 induces apoptosis through specific activation of p53.

Example 11

Induction of P53 Accumulation by Inhibition of MDM2 in Different Cell Lines

The strong activation of p53 in MCF-7 cells by AS5-2 (Figure 9) prompted us to further examine its effect on p53. MCF-7 cells predominantly contain a cytoplasmic form of p53 (Takahashi *et al.*, Mol Carcinog 8:58-66 (1993)) and display predominantly cytoplasmic fluorescence when stained using anti-p53 monoclonal antibody Pab1801. After treatment with 200 nM AS5-2 for 20 hr, many MCF-7 cells showed intense nuclear p53 staining (Figure 11). The parent AS5 oligonucleotide also showed a similar, but weaker, ability to induce p53 accumulation (not shown), and the control oligonucleotide K did not induce p53 (Figure 11). This suggests that nuclear p53 in this cell line is being actively degraded by MDM2, not simply being sequestered into the cytoplasm.

In order to determine whether MDM2 also exhibits a similar role in other tumors containing cytoplasmic p53, the neuroblastoma cell line SK-N-SH was tested. Neuroblastomas rarely have p53 mutations but often contain p53 in the cytoplasm. SK-N-SH cells express cytoplasmic wild-type p53 and exhibit a reduced ability to undergo cell cycle arrest after DNA damage. Moll *et al.*, Proc. Natl. Acad. Sci. U.S.A. 92:4407-4411 (1995); Goldman *et al.*, Am. J. Pathol. 148:1381-1385 (1996). When treated with AS5-2, this cell line also displayed a strong accumulation of nuclear p53

in nearly 100% of the cells (Figure 11). This result suggests that in addition to cytoplasmic sequestration, MDM2-mediated degradation is important for the loss of nuclear p53 in some tumors.

This test was then extended to a wide variety of tumor cell lines with wild-type p53. A total of 24 human tumor cell lines representing 15 different tumor types were treated with AS5-2 and stained for p53 expression. Cells were treated with 200 nM of AS5-2 or K oligonucleotide for 20 hr and p53 level was determined semi-quantitatively by immunofluorescene staining with Pab1801. MDM2 levels in untreated cells were determined by staining with 2A9. The results, as shown in Figure 11 and summarized in Table 2, *infra*, revealed that the low levels of wild-type p53 can be significantly stimulated by AS5-2, resulting in intense nuclear p53 staining. Two non-transformed human cell lines, WI-38 (lung fibroblast) and JeKin (skin fibroblast) also showed strong p53 accumulation after inhibition of MDM2 expression. Therefore, this p53 response due to loss of MDM2 is not unique to tumor cells.

Table 2

Cell line	Origin	MDM2 level	p53	Nuclear p53 level	
				Basal	AS5-2 treated
JEG-3	Choriocarcinoma	++++	Wt	++++	++++
JAR	Choriocarcinoma	++++	Wt	++++	++++
SJSA	Osteocarcoma	++++	Wt	-	++++
LS180	Colon carcinoma	-	Wt	-	++++
HT1080	Fibrosarcoma	+	Wt	+	++++
A172	Glioblastoma	-	Wt	-	++++
U87-MG	Glioblastoma	-	Wt	+	++++
HepG2	Hepatocarcinoma	+	Wt	+	++++

Cell line	Origin	MDM2 level	p53	Nuclear p53 level	
				Basal	AS5-2 treated
SLK	Kaposi sarcoma	-	ND	+	++++
101	Kidney tumor	-	Wt	+	++++
A549	Lung tumor	+	Wt	+	++++
G361	Melanoma	++	Wt	+	++++
SK-N-SH	Neuroblastoma	-	Wt	-	++++
MCF-7	Breast carcinoma	+	Wt	+	++++
U2OS	Osteosarcoma	+	Wt	++	+++
PA-1	Ovarian teratoma	+	Wt/mt	-	++++
Lncap	Prostate carcinoma	-	Wt	+	++++
WI-38	Lung Fibroblast	+	Wt	-	++++
JeKin	Skin fibroblast	-	Wt	+	++++
MDA-MB-231	Breast carcinoma	++	Mt	++++	++++
DLD-1	Colon carcinoma	++	Mt	++++	++++
C33A (HPV-)	Cervical carcinoma	+++	Mt	++++	++++
Hela (HPV+)	Cervical carcinoma	-	Wt	-	-
Caski (HPV+)	Cervical carcinoma	-	Wt	-	-
H1299	Lung tumor	-	Null	-	-

Cell line	Origin	MDM2 level	p53	Nuclear p53 level	
				Basal	AS5-2 treated
SK-N-MC	Neuroblastoma	-	Null	-	-

-: not detectable; +: weak staining in most cells or a subset of cells; ++++: strong staining in most or all cells. ND: not determined.

These results also reproduce our previous observation that in certain tumor cells (JAR, JEG-3) with high levels of stable p53, inhibition of MDM2 expression did not result in a significant increase of p53 level. The coexistence of high levels of p53 and MDM2 suggests that the ability of MDM2 to promote degradation of p53 is lost in these cells.

Treatment of tumor cells with homozygous mutant p53 also did not lead to further accumulation of p53, which was already at a high level. A tumor cell line with both wild-type and mutant p53 alleles (PA-1) also contained inducible p53 and underwent apoptosis after AS5-2 treatment (see below). Finally, treatment of HPV-positive cervical cancer cell lines did not induce p53 accumulation, suggesting that HPV E6-mediated degradation of p53 is independent of MDM2 function.

Example 12

Inhibition of MDM2 Expression Prolonged P53 Half Life

The increase of p53 after inhibition of MDM2 expression can result from an increased rate of p53 synthesis or protein stabilization. To directly test these possibilities, the p53 half-life in AS5-2 treated SJSA cells was determined by a pulse-chase radioactive labeling experiment. SJSA cells have MDM2 gene amplification and exhibit a highly inducible wild-type p53 after AS5-2 treatment (Figure 11, Figure 12A). This cell line does not undergo significant apoptosis after AS5-2 treatment, thus can provide sufficient material for analysis.

SJSA cells were treated with 200 nM AS5-2 for 20 hr and pulse labeled with ^{35}S-methionine for 2 hr. The level of p53 was determined at various times after addition of excess cold methionine to prevent further synthesis of radioactive p53. The result showed that the half life of p53 is increased from ~0.5 hr in untreated SJSA cells to >4 hr in AS5-2 treated cells. Furthermore, the amount of radioactive MDM2 synthesized during the 2 hr pulse labeling period did not differ significantly in treated and untreated cells (Figure 12B). Therefore, the rise in p53 level after inhibition of MDM2 expression appears to be due to the stabilization of p53 and not to its increased synthesis.

Example 13

Inhibition of MDM2 Expression Induces Functional p53

In order to determine whether the p53 protein that accumulates after inhibition of MDM2 is functionally active, a p53-responsive BP100-luciferase reporter plasmid was transfected into several representative cell lines. Stably transfected cells were then treated with AS5-2 or control oligonucleotides. The results showed that a strong induction of p53 transcription function occurs after inhibition of MDM2, demonstrating that the p53 accumulated after inhibition of MDM2 is highly active (Figure 13). The magnitude of p53 transcription activation is consistent with the fact that most of the p53 accumulation occurs in the nucleus, which has a low basal level of p53. The 4 bp mismatch control oligonucleotide has significantly reduced efficiency in activation of p53, particularly at low concentrations.

Example 14

Inhibition of MDM2 Leads to Growth Arrest and Apoptosis

A number of cells lines were treated with 200 nM AS5-2 for 20 hr. A significant amount of cell death was evident in many of the tumor cell lines tested (Figure 14). In several cases, cells

rounded up, displayed membrane ruffling and blebbing characteristic of apoptosis, and detached from the culture surface. Thus, it appears that the level of p53 activation achieved by treatment with AS5-2 is sufficient to induce cell death through apoptosis in some of the cell lines examined.

Some of the tumor cell lines as well as two non-transformed cell lines (WI-38 and Jekin) showed little cell death after a 20 hr AS5-2 treatment. Since p53 activation can lead to apoptosis or cell cycle arrest, dependent on the level of p53 and the status of the cell, several of these cell lines were further tested for growth arrest by AS5-2. Cells were treated with AS5-2 or control oligonucleotides for 20 hr, and DNA synthesis was quantitated by BrdU incorporation. The number of viable cells was determined by incubation with the MTS reagent. The results show that in cell lines that do not undergo significant apoptosis after AS5-2 treatment, the rate of DNA synthesis is reduced (Figure 15). This effect is weaker with a 4 bp mismatch control oligonucleotide and is not observed with the unrelated oligonucleotide K. In contrast, the p53-null cell lines H1299 (human) and 10(1) (mouse) (Harvey and Levine, *Genes. Dev.* **5**, 2375-2385 (1991)) did not undergo significant growth inhibition. Therefore induction of p53 by inhibition of MDM2 can lead to growth arrest or apoptosis.

Example 15

In Vitro Antitumor Activity in Human Colon Cancer Models

Test Oligonucleotides. The test oligonucleotide, Oligo AS, a 20-mer mixed-backbone oligonucleotide (5'-UGACACCTGTTCTCACUCAC-3') and its mismatched control (Oligo ASM, 5'-UGTCACCCTTTTTCATUCAC-3') were synthesized, purified, and analyzed as previously described (Agrawal S. et al., *Proc Natl Acad Sci USA*, **94**: 2620-2625, 1997). Two nucleosides at 5'-end and four nucleosides at 3'-end are 2'-O-methylribonucleosides (represented by boldface letters); the remaining are deoxynucleosides. The underlined

nucleosides of Oligo ASM are the sites of the mismatched controls compared with Oligo AS. For both mixed-backbone oligonucleotides, all internucleotide linkages are phosphorothioate. The purity of the oligonucleotides were shown to be greater than 90% by capillary gel electrophoresis and PAGE, with the remainder being n-1 and n-2 products. The integrity of the internucleotide linkages was confirmed by ^{31}P NMR.

Chemicals and Reagents. Cell culture media and phosphate-buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS), antibodies against p53 and p21, Lipofectin, trypsin, penicillin-streptomycin, and trypan blue stain were purchased from GIBCO-BRL (Grand Island, NY). The anti-human-MDM2 monoclonal antibody (Chen L. et al., *Proc Natl Acad Sci USA*, **95**: 195-200, 1998; Chen L. et al., *Mol. Med.* **5**: 21-34, 1999) was kindly provided by J. Chen (Moffitt Cancer Center, Tampa, FL). Chemotherapeutic agents adriamycin and 5-fluorouracil (5-FU) were obtained from Sigma. Natural product topoisomerase I inhibitor 10-hydroxycamptothecin (HCPT) was obtained from the Midwest Co. (Beijing, China) with the purity of the drug being greater than 98% (Zhang R. et al., *Cancer Chemother Pharm* **41**: 257-267, 1998).

Cell Culture. The tumor cell lines, LS174T and DLD-1 were obtained from the American Type Culture Collection (Rockville, MD) and cultured according to their instructions. LS174T cells were cultured in MEM with 0.1 mM nonessential amino acids and Earle's balanced salt solution containing 10% FBS and DLD-1 cells in RPMI 1640 medium containing 10% FBS. All media included 1% penicillin/streptomycin. *In vitro* biological activity of oligonucleotides was determined by using the conditions described earlier (Chen L. et al., *Proc Natl Acad Sci USA*, **95**: 195-200, 1998; Chen L. et al., *Mol. Med.* **5**: 21-34, 1999; Wang H. et al., *Int J. Oncol.* **15**: 653-660, 1999; Cai Q. et al., *Intl J Oncol*, **10**: 953-960, 1997). Cells were incubated with Oligos AS

or ASM at various concentrations for 72 hr, in the presence of Lipofectin (7 μg/ml). The effects on cell growth were analyzed by trypan blue viability staining (Cai Q. et al., *Intl J Oncol*, **10**: 953-960, 1997).

Western Blot Analysis. The MDM2, p53 and p2l levels in cultured cells were analyzed by using the methods described previously (earlier (Chen L. et al., *Proc Natl Acad Sci USA*, **95**: 195-200, 1998; Chen L. et al., *Mol. Med.* **5**: 21-34, 1999; Wang H. et al., *Int J. Oncol.* **15**: 653-660, 1999). In brief, cell lysate containing identical amounts of total protein were fractionated by SDS-PAGE and transferred to Bio-Rad Trans-Blot nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The nitrocellulose membrane was then incubated with blocking buffer (PBS containing 0.1 % Tween 20 and 5% non-fat milk) for 1 hr at room temperature and washed with the washing buffer (PBS containing 0. 1% Tween 20) for 5 min twice. The membrane was incubated with primary (anti-MDM2, anti-p53, or anti-p21) antibody overnight at 4°C or for 1 hr at room temperature with gentle shaking. The membrane was washed with the washing buffer for 15 min and then twice for 5 min, and then incubated with 1:5000 diluted goat anti-mouse IgG-horse radish peroxidase conjugated antibody (Bio-Rad, Hercules, CA) for 1 hr at room temperature. After washing as described above the protein of interest was detected by ECL reagents from Amersham (Arlington Height, IL).

Results.

LS174T Model. *In vitro* inhibition of MDM2 expression by Oligo AS was shown in a sequence-specific, increasing dose-dependent manner (Fig. 19). Anti-MDM2 oligo, Oligo AS, specifically inhibits MDM2 expression (greater than 90% inhibition observed at 500 nM of Oligo AS) in LS174T cells and p53 and p2l levels elevated accordingly. Control oligonucleotide, Oligo ASM had no effect on MDM2, p53 or p2l protein levels at 500 nM, the

highest concentration tested in the study. Oligo AS inhibited the growth of tumor cell lines *in vitro* in a dose-dependent manner, with IC_{50} being 323 nM. The mismatched oligonucleotide, Oligo ASM, had no significant effect on tumor cell growth.

Following *in vitro* combination treatment of Oligos and chemotherapeutic agents HCPT, adriamycin, and 5-FU, the protein levels of MDM2, p53 and p2l were determined in LS174T cells. Cells were incubated with 400 nM Oligo in the presence of Lipofectin for 24 hr, followed by addition of various concentrations of chemotherapeutic agents and incubation for additional 24 hr.

As illustrated in Fig. 20 (panel 1, lane A), HCPT induced p53, p21, and MDM2 in a dose-dependent manner as we reported in an early study with human breast cancer cell line MCF-7 (Liu W. et al., *Intl J Oncol*, **12**: 793-804, 1998). Following the treatment with Oligo AS, MDM2 expression was inhibited, resulting in significant elevated p53 and p2l levels (panel I, lane B). The mismatched control Oligo ASM showed minimal effect on the protein levels of MDM2, p53 or p2l (panel 1, lane C). Also shown in Fig. 20, adriamycin slightly induced p53, p21 and MDM2 in LS174T cells (panel II, lane A). However, following the treatment with Oligo AS, MDM2 expression was inhibited, resulting in significant elevated p53 and p2l levels (panel II, lane B). The mismatched control Oligo ASM showed minimal effect on the protein levels of MDM2, p53 or p2l (panel II, lane C). The effects of Oligo AS on 5-FU-induced p53, p2l and MDM2 levels were also evaluated (Fig. 20, panel 111). 5-FU induced p53, p21, and MDM2 in a dose-dependent manner (panel 111, lane A). Following the treatment with Oligo AS, MDM2 expression was inhibited, resulting in significant elevated p53 and p2l levels (panel 111, lane B). The mismatched control Oligo ASM showed minimal effect on the protein levels of MDM2, p53 or p2l (panel III, lane C).

DLD-1 Model. *In vitro* inhibition of MDM2 expression by Oligo AS was shown in a sequence-specific, dose-dependent manner (Fig. 23). The best inhibitory effect (>95%) on MDM2 expression in DLD-1 cells was observed with 200 nM Oligo AS in the presence of Lipofectin, whereas the inhibitory effects were approximately 80% at higher concentrations (500 and 1000 nM). No significant changes in the protein levels of the mutant p53 were observed after Oligo AS treatment. The p2l levels were elevated at the concentration of 100 nm or higher, which is independent to p53. Control oligonucleotide, Oligo ASM had no effect on MDM2, p53 or p2l protein levels. Oligo AS inhibited the growth of tumor cell lines *in vitro* in a dose-dependent manner, with IC_{50} being 140 nM in a 72-hr treatment protocol in presence of Lipofectin. The mismatched oligonucleotide, Oligo ASM, had no significant effect on tumor cell growth.

Following *in vitro* combination treatment of Oligos and chemotherapeutic agents HCPT, adriamycin, and 5-FU, the protein levels of MDM2, p53 and p2l were determined in DLD-1 cells. Cells were incubated with 200 nM Oligo in the presence of Lipofetin for 24 hr, followed by addition of various concentrations of chemotherapeutic agents and incubation for an additional 24 hr.

As illustrated in Fig. 24 (panel 1, lane A), HCPT induced MDM2 and p2l in a dose-dependent manner as we reported in an earlier study with human breast cancer cell line MDA-MB-468 that contains mutant p53 (Liu W. et al., *Intl J Oncol*, **12**:793-804, 1998). Following the treatment with Oligo AS, MDM2 expression was inhibited, resulting in significant elevated p2l levels (panel 1, lane B). The mismatched control Oligo ASM showed minimal effect on the protein levels of MDM2 or p2l (panel 1, lane C). No changes in p53 levels in cells were

observed in cells untreated or treated with HCPT in the presence of Lipofectin, Oligo AS or ASM, indicating that the changes in MDM2 and p21 levels were independent to p53 status.

Also shown in Fig. 24, adriamycin induced p21 and MDM2 in DLD- I cells (panel 11, lane A). Following the treatment with Oligo AS, MDM2 expression was inhibited, resulting in significant elevated p21 levels (panel 11, lane B). The mismatched control Oligo ASM showed minimal effect on the protein levels of MDM2 or p21 (panel 11, lane C). 5-FU significantly induced MDM2 in a dose-dependent manner (panel 111, lane A). Following the treatment with Oligo AS, MDM2 expression was inhibited, and p21 slightly induced (panel 111, lane B). The mismatched control Oligo ASM showed minimal effect on the protein levels of MDM2, p53 or p21 (panel IH, lane C). Once again, no changes in p53 levels in cells were observed in cells untreated or treated with adriamycin or 5-FU in presence of Lipofectin, Oligo AS or ASM, indicating that the changes in MDM2 and p21 levels are independent to p53 status.

Example 16

In Vivo Antitumor Activity in Human Colon Cancer Models

Animal Tumor Model. Human cancer xenograft models were established using the methods reported previously (Wang H. et al., *Int J. Oncol.* **15**: 653-660, 1999; Cai Q. et al., *Intl J Oncol*, **10**: 953-960, 1997; Wang H. et al., *Proc Natl Acad Sci USA*, **96**: 13989-13994, 1999). Female nude mice (five weeks old) were purchased from Frederick Cancer Research and Development Center (Frederick, MD) and accommodated for 5 days for environmental adjustment prior to study. Cultured LS174T or DLD-1 cells were harvested from the monolayer cultures, washed twice with culture medium (without FBS), resuspended in FBS-free culture medium, and injected s.c. (2×10^6 cells, total volume 0.2 ml) into the left inguinal area of the mice. The

animals were monitored for general clinical observation, determination of body weight, and measurement of tumor growth.

***In Vivo* Chemotherapy.** The animals bearing human cancer xenografts were randomly divided into various treatment groups and the control group (6 mice/group). The control (non-oligo treated) group received physiological saline only. The oligonucleotides dissolved in physiological saline (0.9% NaCl) were administered by i.p. injection. The injection volume was based on the body weight (5 μl/g body weight) and the oligonucleotide concentrations were adjusted on the basis of the dose. HCPT was suspended in cottonseed oil and given by gavage (volume; 10 μl/g body weight). The dose was 3 mg/kg/day, 7 consecutive days for the first week of treatment and every other day afterwards. 5-FU was given by i.p. injection at the dose of 10 mg/kg/day (volume; 5 μl/g body weight). Tumor growth was monitored by the measurement, with calipers, of two perpendicular diameters of the implant every other day. Tumor weight (g) was calculated by the formula, 1/2a X b^2 where "a" is the long diameter (cm) and "b" is the short diameter (cm).

Western Blot Analysis. This procedure was performed according to the procedure found in Example 15.

Results.

LS174T Model. Based on previous studies with cell lines that contain wild type p53, the effect of Oligo AS on *in vivo* tumor growth was elevated in LS174T xenograft model at a daily ip dose of 20 mg/kg. Oligo AS showed significant inhibitory effect on tumor growth (Fig. 21, Table 3). The mismatched control Oligo ASM showed minimal effect (Fig. 3A, Table 1). Following HCPT treatment (3 mg/kg/day for the first week and 3 mg/kg every other day for remaining treatment period), tumor growth was inhibited by approximately 50% (Fig. 21A and

Table 3). Following the combination treatment of Oligo AS and HCPT, significant synergistic effects were observed (Fig. 21A, and Table 3). At the end of the experiment, the mean tumor size of the animals treated with HCPT and Oligo AS was 12% of that of the control animals treated with saline, whereas the mean tumor sizes for animals treated with Oligo AS or HCPT alone were 37.3% and 50.8% of that of the controls, respectively (Table 3). Similar significant synergistic effects were observed following the combination treatment of Oligo AS and 5-FU (Fig. 21B, and Table 3). At the end of the experiment, the mean tumor size of the animals treated with 5-FU and Oligo AS was 10.9 % of that of the control animals treated with saline, whereas the mean tumor sizes for animals treated with Oligo AS or HCPT alone were 37.3% and 45.3 % of that of the controls, respectively (Table 3). The mismatched control Oligo ASM showed no significant effect on HCPT or 5-FU- associated tumor growth inhibition (Fig. 21, Table 3). The representative animals treated with various protocols and tumor tissues removed from these animals were illustrated in Fig. 22. The results show a significant synergistic effects following the combination treatment of Oligo AS and 5-FU.

Table 3.

Therapeutic Effectiveness of Anti-MDM2 Oligonucleotide Administered Alone or in Combination with Cytotoxic Agents (Growth ratio: % T: C[E])

Day	No Oligo [1] (%)	+ASM[A] [2] (%)	+ASM[A] Ratio (%) ([2]/[1])	+Anti-MDM2 AS[B] [3] (%)	+Anti-MDM2 AS[B] Ratio (%) ([3]/[1])
3					
Oligo alone	100	98.6	99	50.9	51
HCPT[C]	74.4	102.7	138	36.8	49
5-FU[D]	50.1	50.9	102	47.2	94

6						
	Oligo alone	100	104.9	105	44.1	44
	HCPT	41.2	55.1	134	23.7	58
	5-FU	40.2	67.0	167	38.5	96
9						
	Oligo alone	100	69.7	70	29.9	30
	HCPT	30.0	32.0	107	14.9	50
	5-FU	32.5	50.6	156	35.0	108
12						
	Oligo alone	100	81.5	82	34.3	34
	HCPT	43.7	37.3	85	14.9	34
	5-FU	39.7	39.3	99	29.3	73
15						
	Oligo alone	100	79.4	79	45.2	45
	HCPT	49.1	36.7	75	15.6	32
	5-FU	41.0	39.0	95	21.6	53
18						
	Oligo alone	100	73.3	73	37.3	37
	HCPT	50.8	32.3	63	12.0	24
	5-FU	45.3	32.8	72	10.9	24

ASM[A]:Mismatched Control Oligo; AS[B]: Antisense Anti-MDM2 Oligo; HCPT[C]: 10Hydroxycamptothecin; 5-FU[D]: 5- Fluorouracil; % = % T:C[E]: Percentage of mean tumor mass of treated group compared with the control group treated with saline.

The Ratio ([2]/[1] or [3]/[1]) can be used to illustrate the potential additive or synergistic effects when the oligos were given in combination with cytotoxic agents. When the ratio for combination therapy is less than 100% (compared to cytotoxic agents alone), an effect of antisense oligo is indicated. If the ratio for combination therapy is the same as that of oligo treatment alone, an additive effect is indicated. If the ratio for combination therapy is significantly less than that of oligo treatment alone, a synergistic effect is indicated. For example, at the end of the experiment (day 18), the ratio for 5-FU + AS/5-FU is 24%

(10.9%/45.3%) and less than 100%, indicating an effect of oligo AS, and, in addition, this ratio is less than the ratio for oligo AS alone (37%; AS/Saline), indicating a synergistic effect between 5-FU and Oligo AS. The ratio for 5-FU + ASM/5-FU is 72% (32.8%/45.3%) and less than 100%, indicating an effect of oligo ASM, and, however, this ratio is almost the same as the ratio for oligo ASM alone (72%), indicating no synergistic effect, but an additive effect, between 5-FU and Oligo ASM. In conclusion, additive or synergistic effects between HCPT and Oligo AS were found throughout the treatment period. Additive or synergistic effects between 5-FU and Oligo AS were found only on day 15 and 18. No additive or synergistic effects between Oligo ASM and HCPT or 5-FU were found, except for HCPT + ASM on day 15 and 18 and 5-FU + ASM on day 18. An additive effect between HCPF and ASM was found on these days.

Xenograft Pathology. LS174T Xenografts: Tumors from control mice and mice given Oligo ASM were indistinguishable histologically. Cellularity of the tumors is typical of anaplastic colon cancer as evidenced by disorderly gland formation, oval to round open-faced nuclei, some degree of polarity of cells with apical clear zones and basilar nuclei, modest to abundant mucous production and numerous mitotic figures. Tumor tissues from mice treated with Oligo AS contains patches of collagenous connective tissues replacing tumor and necropurulent, ulcerative changes. No significant histological changes were found in tumors of mice treated with 5-FU alone. Tissues from mice treated with combination of 5-FU and Oligo AS were shown to be small nodule of scar tissue which contains small islands of tumor cells. Tumor tissues from mice treated with HCPT alone or in combination with Oligo ASM contain large necropurulent, ulcerative changes. Tumor tissues from mice treated with HCPF in combination with Oligo AS were shown very atrophic changes in tumor histology.

Host Toxicity. One of the concerns of combination therapy is the potential risk in increasing host toxicity. In the present study, no significant increase in host toxicity, in terms of body weight gain/loss and survival rates, was observed in combination treatment (Table 4).

Table 4.

Survival Rates of Animals Treated with Anti-MDM2 Oligonucleotide Administered Alone or in Combination with Cytotoxic Agents

Day AS	No Oligo (%)	+ASM (%)	+Anti-MDM2 [(%)]
3			
Oligo alone	100	100	100
HCPT	100	100	100
5-FU	100	100	100
6			
Oligo alone	100	100	100
HCPT	100	100	100
5-FU	100	100	100
9			
Oligo alone	100	100	100
HCPT	66.7	83.3	83.3
5-FU	100	100	83.3
12			
Oligo alone	100	100	100
HCPT	50.0	83.3	83.3
5-FU	100	100	83.3
15			
Oligo alone	100	100	100
HCPT	50.0	66.7	83.3
5-FU	100	100	83.3
18			
Oligo alone	100	100	100
HCPT	50.0	66.7	66.7
5-FU	100	100	83.3

DLD-1 Model. The effect of Oligo AS on *in vivo* tumor growth was elevated in DLD-1 xenograft model at various daily ip doses (1, 10, 20 mg/kg), and a dose-dependent response was

observed (Fig. 25A). At the dose of 1 mg/kg/day, Oligo AS showed minimal effects on tumor growth. The mismatched control Oligo ASM showed minimal effect at 20 mg/kg/day (Fig. 25B, Table 3). Following HCPT treatment (3 mg/kg/day for the first week and 3 mg/kg every other day for remaining treatment period), tumor growth was inhibited by approximately 51% after 21-day treatment (Fig. 25B and Table 5). Following the combination treatment of Oligo AS and HCPT, significant additive or synergistic effects were observed (Fig. 25B and Table 5). At the end of the experiment, the mean tumor size of the animals treated with HCPT'and Oligo AS was 20% of that of the control animals treated with saline, whereas the mean tumor sizes for animals treated with Oligo AS or HCPT alone were 36.8% and 5 1.0% of that of the controls, respectively (Table 5). Similar significant additive or synergistic effects were observed following the combination treatment of Oligo AS and 5-FU (Fig. 25C and Table 5). At the end of the experiment, the mean tumor size of the animals treated with 5-FU and Oligo AS was 23.1% of that of the control animals treated with saline, whereas the mean tumor sizes for animals treated with Oligo AS or HCPT alone were 36.8% and 44 % of that of the controls, respectively (Table 5). The mismatched control Oligo ASM showed no significant effect on HCPT or 5-FUassociated tumor growth inhibition (Fig. 25B and C, Table 5), further confirming the specificity of Oligo AS as an antisense agent.

Table 5.

Therapeutic Effectiveness of Anti-MDM2 OligonucleotideAdministered Alone or in Combination with Cytotoxic Agents (Growth ratio: % T: C)

Day	No Oligo [1] (%)	+ASM		+Anti-MDM2 AS	
		[2] (%)	Ratio (%) ([2]/[1])	[3] (%)	Ratio (%) ([3]/[1])
3					

	Oligo alone	100	81.5	82	77.3	77
	HCPT	87.7	92.9	106	79.5	91
	5-FU	79.4	85.4	108	73.8	93
6						
	Oligo alone	100	87.5	88	72.0	72
	HCPT	74.0	68.8	93	56.2	76
	5-FU	75.8	85.9	113	64.7	85
9						
	Oligo alone	100	79.3	70	59.2	59
	HCPT	62.3	61.7	99	40.7	65
	5-FU	61.2	64.9	106	58.0	95
12						
	Oligo alone	100	66.4	66	41.2	41
	HCPT	51.4	49.1	96	27.9	54
	5-FU	46.2	54.4	118	38.5	83
15						
	Oligo alone	100	79.3	79	44.4	44
	HCPT	60.8	57.5	95	27.6	45
	5-FU	54.3	50.9	93	42.4	78
18						
	Oligo alone	100	81.0	81	46.7	47
	HCPT	56.3	52.0	92	27.6	49
	5-FU	55.6	46.2	83	33.1	60
21						
	Oligo alone	100	76.0	76	36.8	37
	HCPT	51.0	45.1	88	20.0	39
	5-FU	44.0	42.5	97	23.1	53

Xenograft Pathology. Tumors from control mice (saline or Oligo ASM) were composed of very tightly packed cells with round to oval, open-faced nuclei and modest amounts of cytoplasm, forming sheets and cords of cells separated by very thin zones of fibrous connective tissue. Although the tumor mass from mice treated with chemotherapeutic agents HCPT and 5-FU alone or in combination with Oligo As were significantly smaller than control tumors, they were histologically similar to the control tumors from mice. Tumors from mice treated with

Oligo AS were histologically similar to control tumors except for modest increases in connective tissue with necrotic changes in tumor tissues.

Host Toxicity. In the present study, no significant increase in host toxicity, in terms of body weight gain/loss and survival rates, was observed in combination treatment of Oligo AS and HCPT or 5-FU (Table 6). Of note, in the combination treatment with HCPT and Oligo ASM, the survival rates were lower than the controls, which, however, is not significantly different from that of the control groups in the study of LS174T model (Table 4). Therefore, the host toxicity observed in the groups treated with the combination protocol (HCPT plus Oligo ASM or AS) may be associated with the cytotoxic agent HCPT rather than Oligos (Tables 4 and 6).

Table 6.

Survival Rates of Animals Treated with Anti-MDM2 Oligonucleotide Administered Alone or in Combination with Cytotoxic Agents

Day AS	No Oligo (%)	+ASM (%)	+Anti-MDM2 [(%)]
3			
Oligo alone	100	100	100
HCPT	100	100	100
5-FU	100	100	100
6			
Oligo alone	100	100	100
HCPT	100	100	83.3
5-FU	100	100	100
9			
Oligo alone	100	100	100
HCPT	100	66.7	83.3
5-FU	100	100	100
12			
Oligo alone	100	100	100
HCPT	100	50.0	83.3
5-FU	100	100	100

15			
Oligo alone	100	100	100
HCPT	100	50.0	83.3
5-FU	100	100	100
18			
Oligo alone	100	100	100
HCPT	100	50.0	83.3
5-FU	100	100	100
21			
Oligo alone	100	66.7	100
HCPT	100	50.0	83.3
5-FU	100	100	100

Conclusions: In LS174T cells that contain wild type p53, cancer chemotherapeutic agents HCPT, adriamycin, and 5-FU induced p53 levels (Fig 20), which, however, was limited due to MDM2 overexpression (Fig. 20). Following treatment with antisense anti-MDM2 Oligo AS, MDM2 expression was specifically inhibited, resulting in significant increases in cytotoxic agent-induced p53 and p21 levels (Fig. 20). These findings are inconsistent with the *in vivo* synergistic effects on antitumor activity following combination treatment of Oligo AS and cytotoxic agents HCPT or 5FU (Fig. 21). These results further confirm our early findings with cell lines that contain amplified MDM2 gene and overexpressed MDM2 protein. Therefore, we conclude that MDM2-p53 interaction can serve as a novel drug target, even MDM2 and/or p53 are expressed in basal levels.

In DLD-1 cells that contain mutant p53, Oligo AS or cancer chemotherapeutic agents HCPT, adriamycin, and 5-FU had no effect on the mutant p53 levels (Fig. 23, 24). These cytotoxic agents, however, induced MDM2 levels although the mechanism is not clear (Fig. 24). These cytotoxic agents also slightly induced p21 levels (Fig. 24). Following treatment with antisense anti-MDM2 Oligo AS, MDM2 expression was specifically inhibited, resulting in

significant increase in cytotoxic agent (HCPT and adriamycin)-induced p21 levels (Fig. 24). More important, *in vivo* antitumor activity of Oligo AS in the DLD-1 model following administration alone or in combination with cytotoxic agents HCPT or 5-FU were observed, which is independent to p53 status (Fig. 25 and Table 5).

In conclusion, we have demonstrated that the selected specific anti-human-MDM2 mixed-backbone oligonucleotide has significant anti-tumor activity *in vitro* and *in vivo*, regardless of p53 status. These results suggest that MDM2 play a role in tumor growth through both p53-dependent and p53-independent mechanisms.

Example 17

In Vivo Studies of Anti-MDM2 Antisense Oligonucleotides

A new generation of mixed-backbone oligonucleotide was designed with the same sequence as AS5-2 and used in *in vivo* studies. The structures of these oligos are illustrated below in Table 7; all internucleotide linkages are phosphorothioates and the underlined nucleotides are 2′-O-methyl substituted.

Table 7

Name	SEQ ID NO:	Sequence
AS5-2	28	5′-TGA CAC CTG TTC TCA CTC AC-3′
AS5-2H	47	5′-<u>UGA</u> CAC CTG TTC TCA <u>CUC AC</u>-3′
AS5-2HM	48	5′-<u>UGA</u> GAC CAG TTG TCA <u>GUC AC</u>-3′

Compared with PS-oligos, hybrid oligos have increased *in vivo* stability, decreased degradation rate, less host toxicity, and, more importantly, increased therapeutic effects (Zhang *et al.*, *Biochem. Pharm.* **49**:929-939, 1995); Zhang *et al.*, *Biochem. Pharm.* **<u>50</u>**:545-556, 1995;

Agrawal and Zhang in Antisense research and Applications, pp. 525-543, S. Crooke, ed., Springer-Verlag, Heidelberg, 1997; and Zhao *et al.*, *Biochem. Pharm.* **51**:173-182, 1996). Therefore, we anticipated that AS5-2H would have a better therapeutic effect *in vivo* than its PS-oligonucleotide counterpart. The tumor cell lines, SJSA and JAR, were cultured under the same conditions as in *in vitro* studies (Chen *et al.*, *PNAS* 1998, *supra*). Human cancer xenograft models were established using the methods reported previously (Cai *et al.*, *Intl. J. Oncol.* **10**:953-960, 1997; and Zhang *et al.*, *Intl. J. Oncol.* **10**:1147-1156, 1997). Female nude mice (five week old) were used in the study. Cultured SJSA and JAR cells were harvested from the monolayer cultures, washed twice with DMEMF-12 HAM medium, resuspended in DMEM, and injected s.c. (20 x 106 cells, total volume 0.2 ml) into the left inguinal area of the mice. The animals were monitored by general clinical observation, body weight, and tumor growth. The animals with SJSA xenografts were used in the chemotherapy study when the tumor size reached 150 mg. Animals with JAR cells were treated when the tumor size reached 2,000 mg. The animals bearing human cancer carcinoma xenografts were randomly divided into treatment groups and a control group (6-10 mice/group). Oligonucleotides dissolved in physiological saline (0.9% NaCl) were administered by ip injection at various daily doses, 5 consecutive days per week. The control group received physiological saline only.

To determine the potential synergistic effects between MDM2 inhibition and DNA damage, oligonucleotides and HCPT were co-administered to tumor bearing mice. Tumor growth was monitored using the methods previously reported (74,75). Tumor weight (g) was calculated by the formula, $\frac{1}{2} \cdot a \cdot b^2$, where "a" is the long diameter (cm) and "b" is the short diameter (cm) of the tumor. At the end of the experiment, tumors were removed, weighed, and then fixed for pathology evaluation.

Fig 16 illustrates the data on *in vivo* anti-tumor activities of antisense anti-MDM2 oligo AS5-2H administered alone and in combination with HCPT, a DNA damaging agent, into mice bearing SJSA xenografts. At the end of the experiment, tumors were removed and weighed. Fig. 17 illustrates representative tumors from various groups. The results from this study can be summarized as follows: 1) control oligonucleotide AS5-2HM had no effect on tumor growth; 2) AS5-2H had a dose-dependent effect on tumor growth; 3) HCPT had a dose-dependent effect on tumor growth; and 4) co-administration of AS5-2H and HCPT had synergistic effects on tumor growth, but no synergistic effect is seen with control oligo AS5-2HM.

In the studies with JAR xenografts, we took a different approach to investigate the effect of anti-MDM2 oligonucleotides on tumor regression and animal survival. In this case, we directly injected the oligonucleotide AS5-2H or HCPT into large tumors (average 2,000 mg), mimicking the clinical late stage of tumors. The results are depicted in Fig. 18. All control animals died within a week after beginning of treatment. HCPT alone had no effect. 20% of animals treated with anti-MDM2 oligonucleotide survived up to 4 weeks, accompanied by tumor regression. Combination treatment of the anti-MDM2 oligonucleotide and HCPT significantly improved the survival rate: 50% of the animals survived over six weeks with almost complete tumor regression. No significant host toxicity was observed. These results further demonstrate that MDM2 inhibition directly correlates with tumor regression and animal survival.

This is the first direct experimental evidence demonstrating a therapeutic effect by an anti-MDM2 antisense oligonucleotide administered alone or in combination with a DNA damaging agent. These data confirm the findings of the previously presented *in vitro* studies.

The invention and the manner and process of making and using it, are now described in such full, clear, concise and exact terms as to enable any person skilled in the art to which it pertains, to make and use the same. It is to be understood that the foregoing describes preferred embodiments of the present invention and that modifications may be made therein without departing from the spirit or scope of the present invention as set forth in the claims. To particularly point out and distinctly claim the subject matter regarded as invention, the following claims conclude this specification.

WHAT IS CLAIMED IS:

1. A method of inhibiting expression of MDM2 in a mammal, the method comprising administering to the mammal an effective MDM2-expression inhibiting amount of an anti-MDM2 antisense oligonucleotide.

2. The method according to claim 1 comprising co-administering a cancer chemotherapeutic agent.

3. The method according to claim 2, wherein the cancer chemotherapeutic agent is 10-hydroxycamptothecin, adriamycin, or 5-fluorouracil.

4. The method according to claim 1 comprising co-treating the mammal with anti-cancer levels of radiation.

5. A method of inhibiting cancer *in vivo*, the method comprising administering a cancer-inhibiting amount of an anti-MDM2 antisense oligonucleotide, wherein the cancer involves overexpression of MDM2.

6. The method according to claim 5, wherein the cancer is selected from the group consisting of osteosarcoma, soft tissue sarcoma, breast cancer, ovarian cancer, cervical cancer, oral squamous cell carcinoma, brain tumor, esophageal cancer, colorectal carcinoma, baldder cancer, urithelial carcinoma, leukemia, and large B cell lymphoma.

7. The method according to claim 5 comprising co-administering an effective cancer-treating amount of a cancer chemotherapeutic agent.

8. The method according to claim 7, wherein the cancer chemotherapeutic agent is 10-hydroxycamptothecin, adriamycin, or 5-fluorouracil.

9. The method according to claim 5 comprising co-treating the mammal with anti-cancer levels of radiation.

10. A method of increasing intracellular p53 concentration, the method comprising administering to the cell or to an animal comprising the cell an effective MDM2-expression inhibiting amount of an anti-MDM2 antisense oligonucleotide.

11. The method according to claim 10 comprising co-administering a cancer chemotherapeutic agent.

12. The method according to claim 11, wherein the cancer chemotherapeutic agent is 10-hydroxycamptothecin, adriamycin, or 5-fluorouracil.

13. The method according to claim 10 comprising co-treating the mammal with anti-cancer levels of radiation.

14. The method according to claim 1, 2, 4, 5, 6, 7, 9, 10, 11, or 13, wherein the antisense oligonucleotide binds to mdm2-encoding RNA is complementary to a sequence that overlaps by at least one nucleotide a sequence within the mdm2 RNA, which sequence within the mdm2 RNA is selected from the group consisting of SEQ ID NOS: 2, 3, 4, 7, 8, 9, 10, and 11, and wherein the antisense oligonucleotide comprises from about 8 to about 50 nucleotides.

15. The method according to claim 1, 2, 4, 5, 6, 7, 9, 10, 11, or 13, wherein the antisense oligonucleotide binds to mdm2-encoding RNA is complementary to a sequence that overlaps by at least one nucleotide a sequence within the mdm2 RNA, which sequence within the mdm2 RNA is selected from the group consisting of SEQ ID NOS: 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, and 24, and wherein the antisense oligonucleotide comprises from about 8 to about 50 nucleotides.

16. The method according to claim 14, wherein the antisense oligonucleotide has a nucleotide base sequence set forth in Sequence Listing as SEQ ID NO:28.

17. The method according to claim 15, wherein the antisense oligonucleotide has a nucleotide base sequence set forth in Sequence Listing as SEQ ID NO:36.

18. The method according to claim 14, wherein the antisense oligonucleotide has a nucleotide base sequence set forth in Sequence Listing as SEQ ID NO: 27, 28, 29, 30, 31, 32, 33, and 34.

19. The method according to claim 15, wherein the antisense oligonucleotide has a nucleotide base sequence set forth in Sequence Listing as SEQ ID NOS: 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, and 46.

20. The oligonucleotide according to claim 14, wherein the oligonucleotide has at least one internucleotide linkage selected from the group consisting of phosphorothioate, phosphorodithioate, alkylphosphonate, alkylphosphonothioate, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate and sulfone internucleotide linkages.

21. The oligonucleotide according to claim 15, wherein the oligonucleotide has at least one internucleotide linkage selected from the group consisting of phosphorothioate, phosphorodithioate, alkylphosphonate, alkylphosphonothioate, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate and sulfone internucleotide linkages.

22. The method according to claim 20, wherein the antisense oligonucleotide comprises an RNase H activating segment of four or more consecutive phosphodiester and/or phosphorothioate internucleotide linkages.

23. The method according to claim 21, wherein the antisense oligonucleotide comprises an RNase H activating segment of four or more consecutive phosphodiester and/or phosphorothioate internucleotide linkages.

24. The method according to claim 22, wherein the RNase H activating segment is flanked on both sides by a segment of two or more nucleotides that are modified to increase nuclease resistance and/or target hybridization affinity.

25. The method according to claim 23, wherein the RNase H activating segment is flanked on both sides by a segment of two or more nucleotides that are modified to increase nuclease resistance and/or target hybridization affinity.

26. The method according to claim 24, wherein the nucleotides of the segments of 2 or more nucleotides are 2′-substituted ribonucleotides.

27. The method according to claim 25, wherein the nucleotides of the segments of 2 or more nucleotides are 2′-substituted ribonucleotides.

28. The method according to claim 26, wherein the 2′-substituted nucleotides are substituted at their 2′ position with methoxy or methoxyethoxy.

29. The method according to claim 27, wherein the 2′-substituted nucleotides are substituted at their 2′ position with methoxy or methoxyethoxy.

ABSTRACT OF THE DISCLOSURE

The invention provides methods to activate tumor suppressors. The invention further provides antisense oligonucleotides complementary to a portion of the MDM2-encoding RNA and methods for using such antisense oligonucleotides as analytical and diagnostic tools, as potentiators of transgenic animal studies and for gene therapy approaches, and as potential therapeutic agents. The invention also provides methods to augment and synergistically activate a tumor suppressor in conjunction with the use of a DNA-damage inducing agent. The invention further provides *in vitro* and *in vivo* models to evaluate the therapeutic effectiveness of a recently identified anti-human-MDM2 antisense oligonucleotide in the treatment of human colorectal cancers.

GCACCGCGCG AGCTTGGCTG CTTCTGGGGC CTGTGTGGCC CTGTGTGTCG GAAAGATGGA
GCAAGAAGCC GAGCCCGAGG GGCGGCCGCG ACCCCTCTGA CCGAGATCCT GCTGCTTTCG
CAGCCAGGAG CACCGTCCCT CCCCGGATTA GTGCGTACGA GCGCCCAGTG CCCTGGCCCG
GAGAGTGGAA TGATCCCCGA GGCCCAGGGC GTCGTGCTTC CGCAGTAGTC AGTCCCCGTG
AAGGAAACTG GGGAGTCTTG AGGGACCCCC GACTCCAAGC GCGAAAACCC CGGATGGTGA
GGAGCAGGCA AATGTGCAAT ACCAACATGT CTGTACCTAC TGATGGTGCT GTAACCACCT
CACAGATTCC AGCTTCGGAA CAAGAGACCC TGGTTAGACC AAAGCCATTG CTTTTGAAGT
TATTAAAGTC TGTTGGTGCA CAAAAAGACA CTTATACTAT GAAAGAGGTT CTTTTTTATC
TTGGCCAGTA TATTATGACT AAACGATTAT ATGATGAGAA GCAACAACAT ATTGTATATT
GTTCAAATGA TCTTCTAGGA GATTTGTTTG GCGTGCCAAG CTTCTCTGTG AAAGAGCACA
GGAAAATATA TACCATGATC TACAGGAACT TGGTAGTAGT CAATCAGCAG GAATCATCGG
ACTCAGGTAC ATCTGTGAGT GAGAACAGGT GTCACCTTGA AGGTGGGAGT GATCAAAAGG
ACCTTGTACA AGAGCTTCAG GAAGAGAAAC CTTCATCTTC ACATTTGGTT TCTAGACCAT
CTACCTCATC TAGAAGGAGA GCAATTAGTG AGACAGAAGA AAATTCAGAT GAATTATCTG
GTGAACGACA AAGAAAACGC CACAAATCTG ATAGTATTTC CCTTTCCTTT GATGAAAGCC
TGGCTCTGTG TGTAATAAGG GAGATATGTT GTGAAAGAAG CAGTAGCAGT GAATCTACAG
GGACGCCATC GAATCCGGAT CTTGATGCTG GTGTAAGTGA ACATTCAGGT GATTGGTTGG
ATCAGGATTC AGTTTCAGAT CAGTTTAGTG TAGAATTTGA AGTTGAATCT CTCGACTCAG
AAGATTATAG CCTTAGTGAA GAAGGACAAG AACTCTCAGA TGAAGATGAT GAGGTATATC
AAGTTACTGT GTATCAGGCA GGGGAGAGTG ATACAGATTC ATTTGAAGAA GATCCTGAAA
TTTCCTTAGC TGACTATTGG AAATGCACTT CATGCAATGA AATGAATCCC CCCCTTCCAT
CACATTGCAA CAGATGTTGG GCCCTTCGTG AGAATTGGCT TCCTGAAGAT AAAGGGAAAG
ATAAAGGGGA AATCTCTGAG AAAGCCAAAC TGGAAAACTC AACACAAGCT GAAGAGGGCT
TTGATGTTCC TGATTGTAAA AAAACTATAG TGAATGATTC CAGAGAGTCA TGTGTTGAGG
AAAATGATGA TAAAATTACA CAAGCTTCAC AATCACAAGA AAGTGAAGAC TATTCTCAGC
CATCAACTTC TAGTAGCATT ATTTATAGCA GCCAAGAAGA TGTGAAAGAG TTTGAAAGGG
AAGAAACCCA AGACAAAGAA GAGAGTGTGG AATCTAGTTT GCCCCTTAAT GCCATTGAAC
CTTGTGTGAT TTGTCAAGGT CGACCTAAAA ATGGTTGCAT TGTCCATGGC AAAACAGGAC
ATCTTATGGC CTGCTTTACA TGTGCAAAGA AGCTAAAGAA AAGGAATAAG CCCTGCCCAG
TATGTAGACA ACCAATTCAA ATGATTGTGC TAACTTATTT CCCCTAGTTG ACCTGTCTAT
AAGAGAATTA TATATTTCTA ACTATATAAC CCTAGGAATT TAGACAACCT GAAATTTATT
CACATATATC AAAGTGAGAA AATGCCTCAA TTCACATAGA TTTCTTCTCT TTAGTATAAT
TGACCTACTT TGGTAGTGGA ATAGTGAATA CTTACTATAA TTTGACTTGA ATATGTAGCT
CATCCTTTAC ACCAACTCCT AATTTTAAAT AATTTCTACT CTGTCTTAAA TGAGAAGTAC
TTGGTTTTTT TTTTCTTAAA TATGTATATG ACATTTAAAT GTAACTTATT ATTTTTTTTG
AGACCGAGTC TTGCTCTGTT ACCCAGGCTG GAGTGCAGTG GGTGATCTTG GCTCACTGCA
AGCTCTGCCC TCCCCGGGTT CGCACCATTC TCCTGCCTCA GCCTCCCAAT TAGCTTGGCC
TACAGTCATC TGCCACCACA CCTGGCTAAT TTTTTGTACT TTTAGTAGAG ACAGGGTTTC
ACCGTGTTAG CCAGGATGGT CTCGATCTCC TGACCTCGTG ATCCGCCCAC CTCGGCCTCC
CAAAGTGCTG GGATTACAGG CATGAGCCAC CG

FIG. 1A

GAGGAGCCGC CGCCTTCTCG TCGCTCGAGC TCTGGACGAC CATGGTCGCT CAGGCCCCGT
CCGCGGGGCC TCCGCGCTCC CCGTGAAGGG TCGGAAGATG CGCGGGAAGT AGCAGCCGTC
TGCTGGGCGA GCGGGAGACC GACCGGACAC CCCTGGGGGA CCCTCTCGGA TCACCGCGCT
TCTCCTGCGG CCTCCAGGCC AATGTGCAAT ACCAACATGT CTGTGTCTAC CGAGGGTGCT
GCAAGCACCT CACAGATTCC AGCTTCGGAA CAAGAGACTC TGGTTAGACC AAAACCATTG
CTTTTGAAGT TGTTAAAGTC CGTTGGAGCG CAAAACGACA CTTACACTAT GAAAGAGATT
ATATTTTATA TTGGCCAGTA TATTATGACT AAGAGGTTAT ATGACGAGAA GCAGCAGCAC
ATTGTGTATT GTTCAAATGA TCTCCTAGGA GATGTGTTTG GAGTCCCGAG TTTCTCTGTG
AAGGAGCACA GGAAAATATA TGCAATGATC TACAGAAATT TAGTGGCTGT AAGTCAGCAA
GACTCTGGCA CATCGCTGAG TGAGAGCAGA CGTCAGCCTG AAGGTGGGAG TGATCTGAAG
GATCCTTTGC AAGCGCCACC AGAAGAGAAA CCTTCATCTT CTGATTTAAT TTCTAGACTG
TCTACCTCAT CTAGAAGGAG ATCCATTAGT GAGACAGAAG AGAACACAGA TGAGCTACCT
GGGGAGCGGC ACCGGAAGCG CCGCAGGTCC CTGTCCTTTG ATCCGAGCCT GGGTCTGTGT
GAGCTGAGGG AGATGTGCAG CGGCGGCACG AGCAGCAGTA GCAGCAGCAG CAGCGAGTCC
ACAGAGACGC CCTCGCATCA GGATCTTGAC GATGGCGTAA GTGAGCATTC TGGTGATTGC
CTGGATCAGG ATTCAGTTTC TGATCAGTTT AGCGTGGAAT TTGAAGTTGA GTCTCTGGAC
TCGGAAGATT ACAGCCTGAG TGACGAAGGG CACGAGCTCT CAGATGAGGA TGATGAGGTC
TATCGGGTCA CAGTCTATCA GACAGGAGAA AGCGATACAG ACTCTTTTGA AGGAGATCCT
GAGATTTCCT TAGCTGACTA TTGGAAGTGT ACCTCATGCA ATGAAATGAA TCCTCCCCTT
CCATCACACT GCAAAAGATG CTGGACCCTT CGTGAGAACT GGCTTCCAGA CGATAAGGGG
AAAGATAAAG TGGAAATCTC TGAAAAAGCC AAACTGGAAA ACTCAGCTCA GGCAGAAGAA
GGCTTGGATG TGCCTGATGG CAAAAAGCTG ACAGAGAATG ATGCTAAAGA GCCATGTGCT
GAGGAGGACA GCGAGGAGAA GGCCGAACAG ACGCCCCTGT CCCAGGAGAG TGACGACTAT
TCCCAACCAT CGACTTCCAG CAGCATTGTT TATAGCAGCC AAGAAAGCGT GAAAGAGTTG
AAGGAGGAAA CGCAGCACAA AGACGAGAGT GTGGAATCTA GCTTCTCCCT GAATGCCATC
GAACCATGTG TGATCTGCCA GGGGCGGCCT AAAAATGGCT GCATTGTTCA CGGCAAGACT
GGACACCTCA TGTCATGTTT CACGTGTGCA AAGAAGCTAA AAAAAAGAAA CAAGCCCTGC
CCAGTGTGCA GACAGCCAAT CCAAATGATT GTGCTAAGTT ACTTCAACTA GCTGACCTGC
TCACAAAAAT AGAATTTTAT ATTTCTAACT

FIG. 1B

FIG. 2A

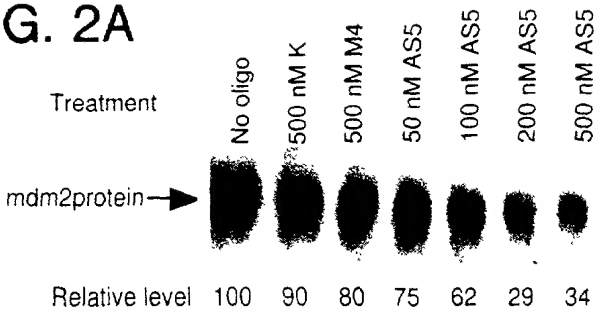


FIG. 2B

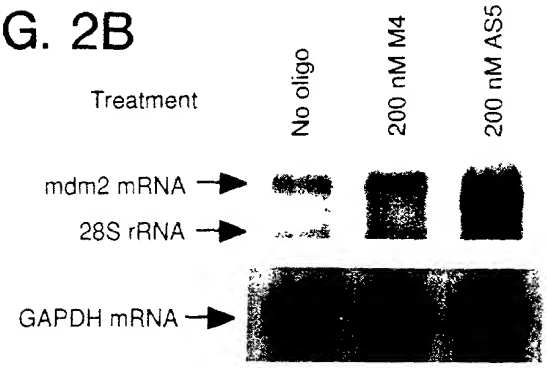
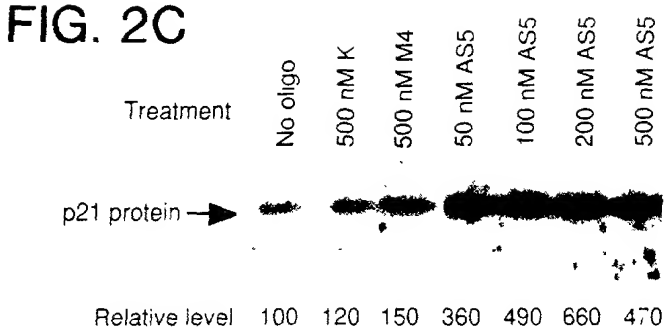


FIG. 2C



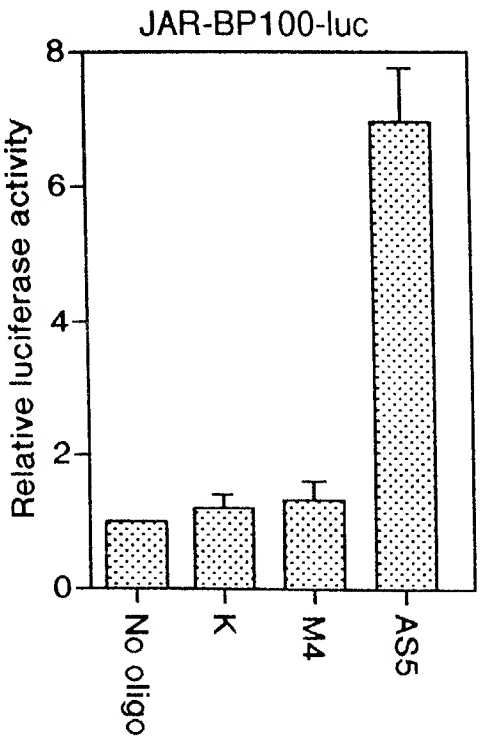


FIG. 3A

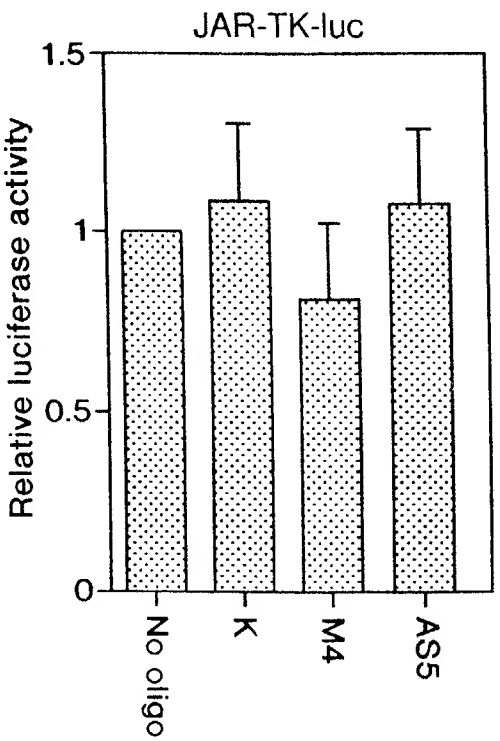


FIG. 3B

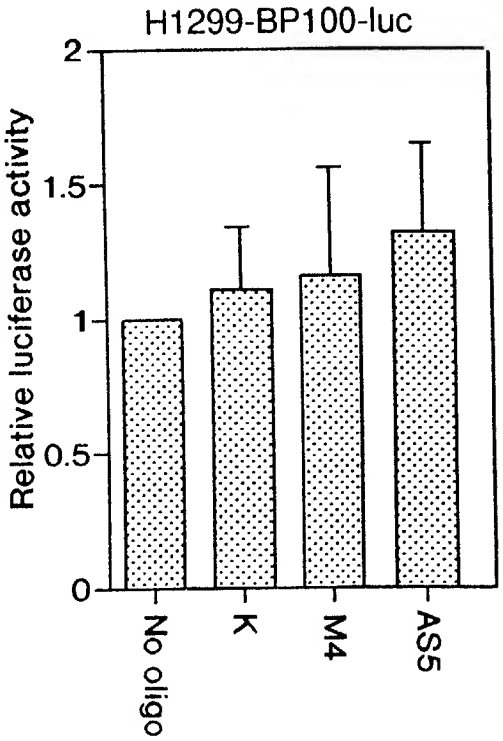


FIG. 3C

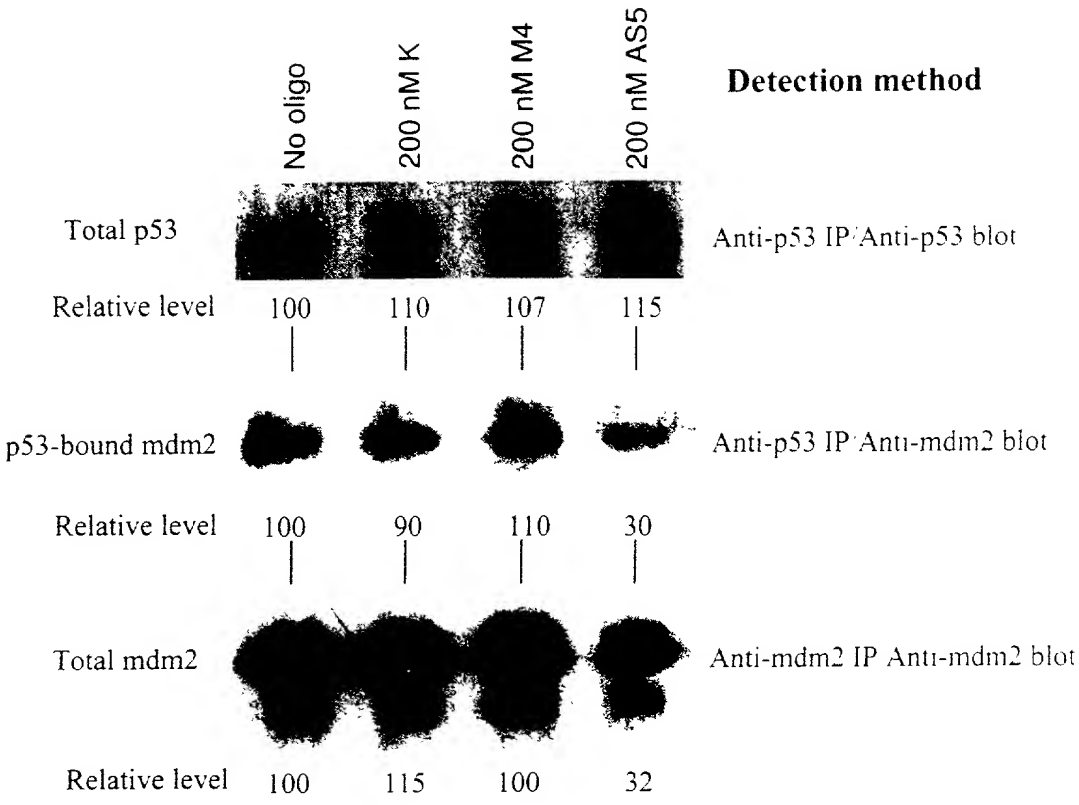

No oligo
200 nM K
200 nM M4
200 nM AS5
Detection method
Total p53
Anti-p53 IP/Anti-p53 blot
Relative level
100
110
107
115
p53-bound mdm2
Anti-p53 IP/Anti-mdm2 blot
Relative level
100
90
110
30
Total mdm2
Anti-mdm2 IP Anti-mdm2 blot
Relative level
100
115
100
32

FIG. 4

JAR + 200 nM AS5

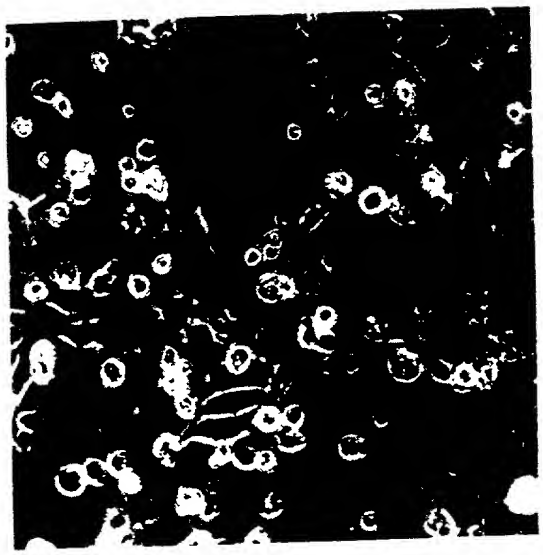


FIG. 5A

JAR + 200 nM M4

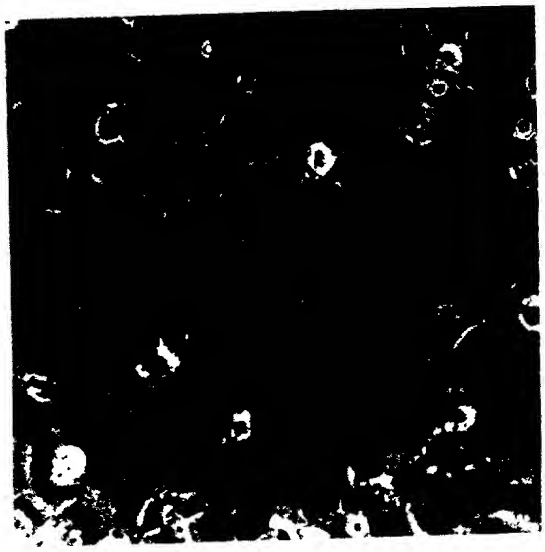


FIG. 5B

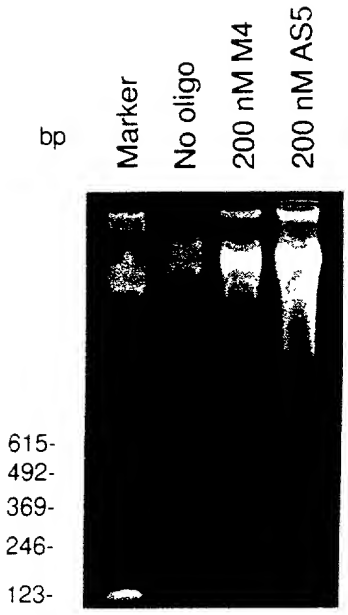


FIG. 6

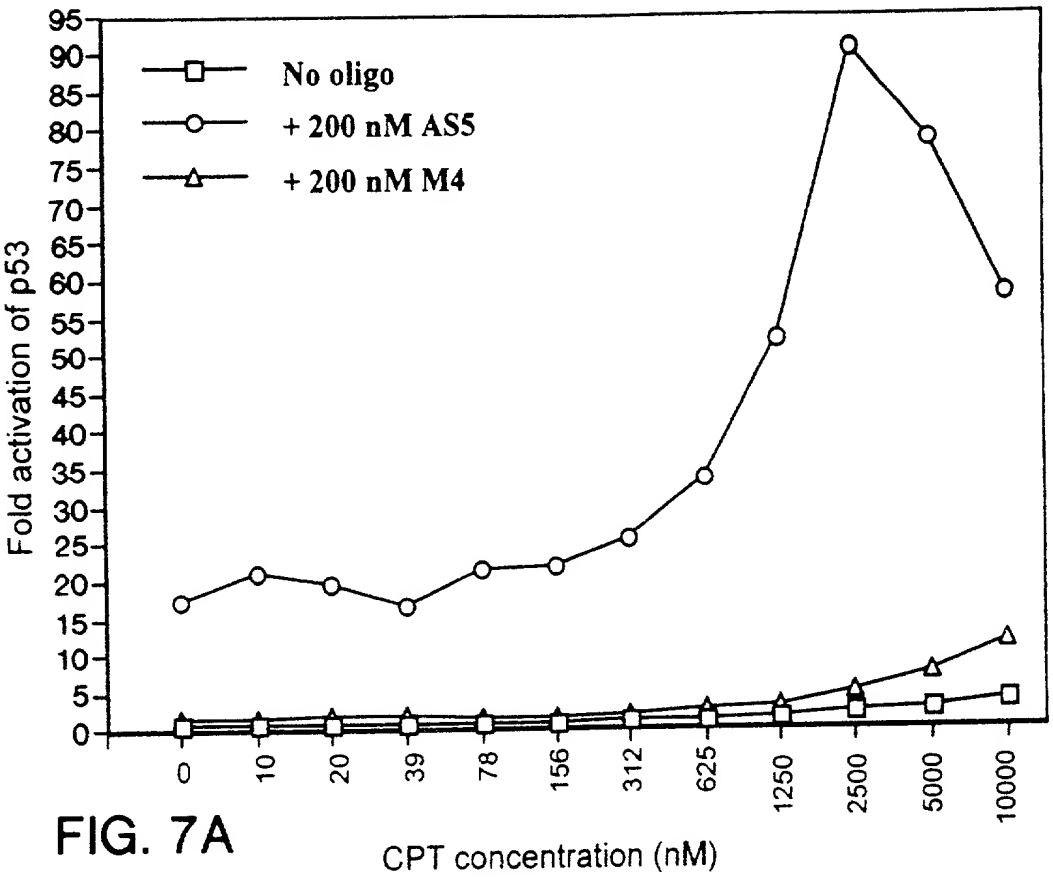

No oligo
+ 200 nM AS5
+ 200 nM M4
Fold activation of p53
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0
10
20
39
78
156
312
625
1250
2500
5000
10000
CPT concentration (nM)

FIG. 7A

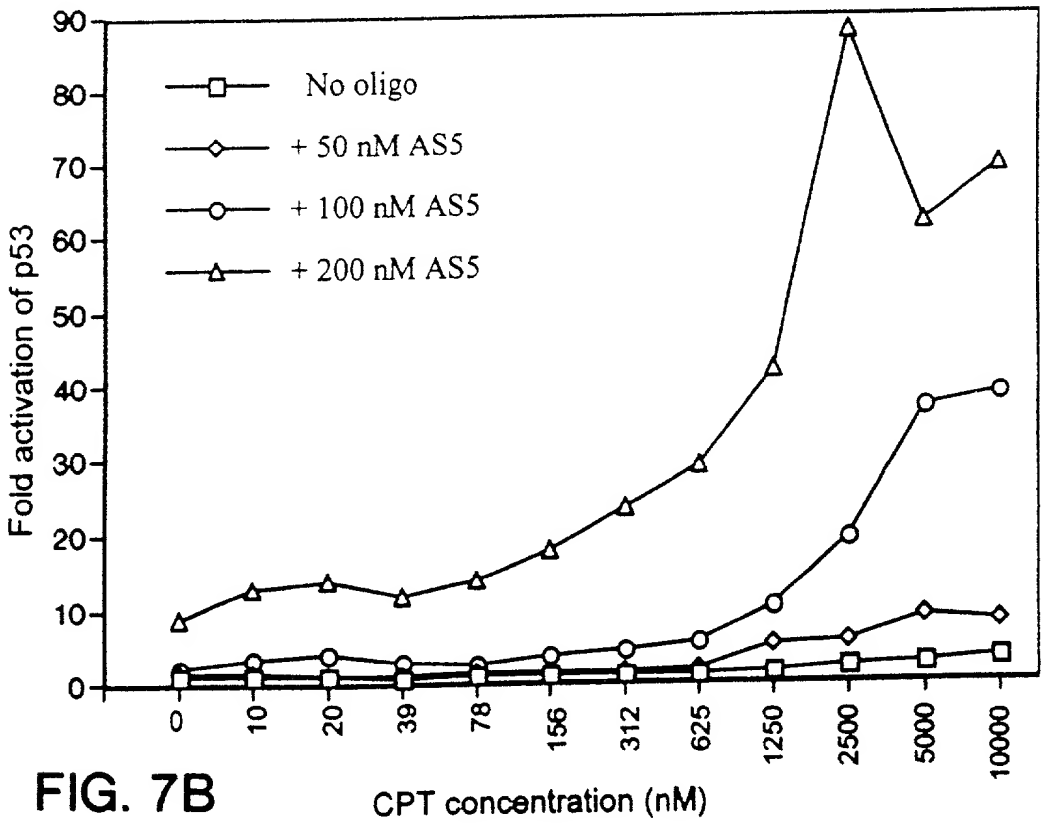

No oligo
+ 50 nM AS5
+ 100 nM AS5
+ 200 nM AS5
Fold activation of p53
90
80
70
60
50
40
30
20
10
0
0
10
20
39
78
156
312
625
1250
2500
5000
10000
CPT concentration (nM)

FIG. 7B

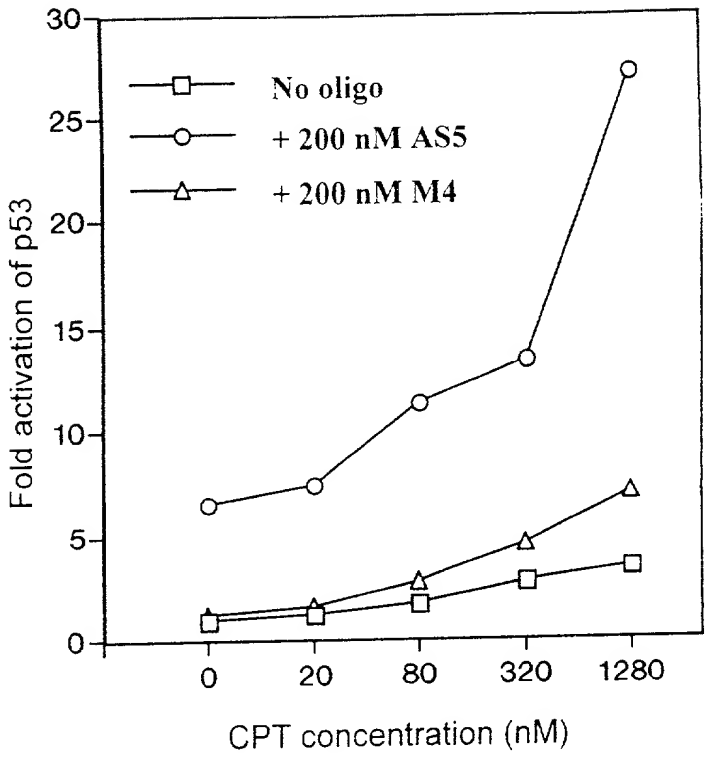

30
25
20
15
10
5
0
Fold activation of p53
No oligo
+ 200 nM AS5
+ 200 nM M4
0
20
80
320
1280
CPT concentration (nM)

FIG. 7C

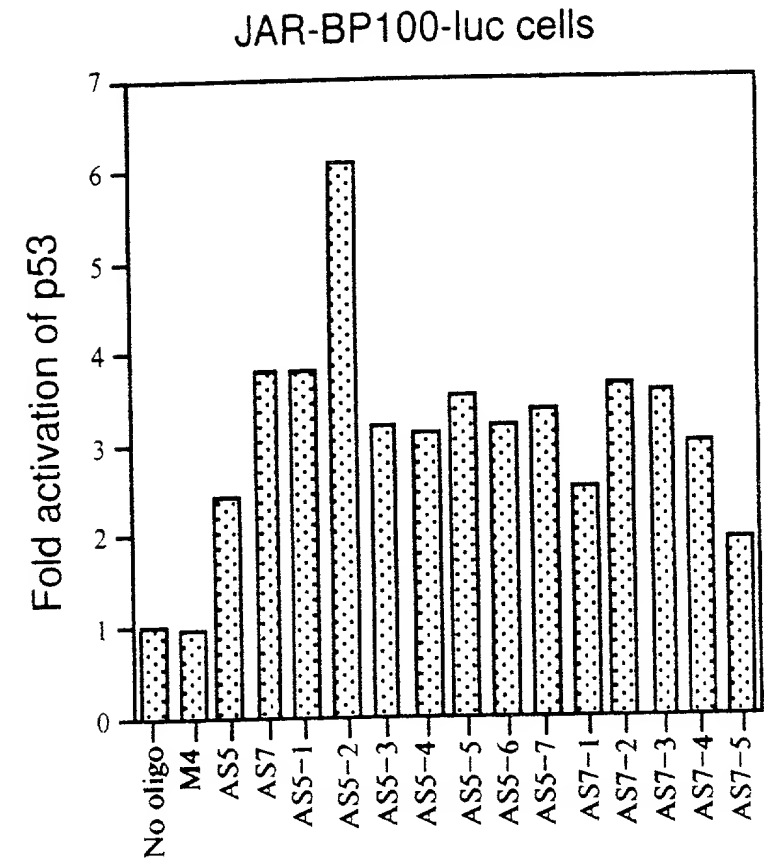

JAR-BP100-luc cells
Fold activation of p53
0
1
2
3
4
5
6
7
No oligo
M4
AS5
AS7
AS5-1
AS5-2
AS5-3
AS5-4
AS5-5
AS5-6
AS5-7
AS7-1
AS7-2
AS7-3
AS7-4
AS7-5

FIG. 8A

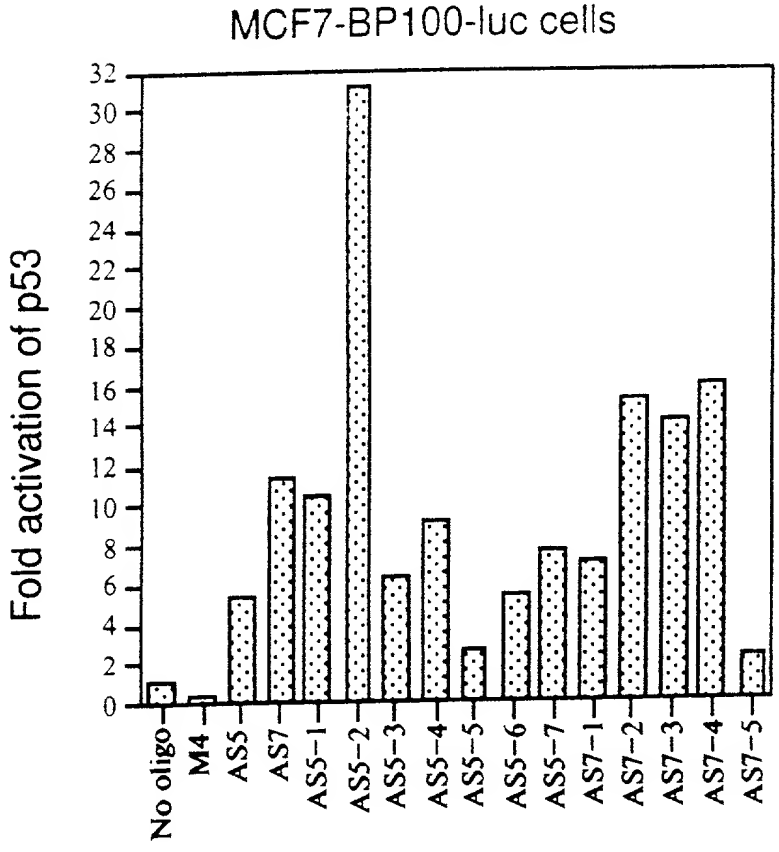

MCF7-BP100-luc cells
Fold activation of p53
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
No oligo
M4
AS5
AS7
AS5-1
AS5-2
AS5-3
AS5-4
AS5-5
AS5-6
AS5-7
AS7-1
AS7-2
AS7-3
AS7-4
AS7-5

FIG. 8B

353-TCCATGTAGACACTCACTCTTGTCCACAGTGGAACTTCCACCCTCACTAGTTTTCCTGGAACATGTTCTCGAA-425 SEQ ID NO: 49

SEQ ID NO.: 35 **AS5-1**: TGTAGACACTCACTCTTGTC **AS5**: GGAACTTCCACCCTCACTAG SEQ ID NO.: 28

SEQ ID NO.: 36 **AS5-2:** CACTCACTCTTGTCCACAGT **AS5-5**: ACCCTCACTAGTTTTCCTGG SEQ ID NO.: 39

SEQ ID NO.: 37 **AS5-3**: ACTCTTGTCCACAGTGGAAC **AS5-6**: CACTAGTTTTCCTGGAACAT SEQ ID NO.: 40

SEQ ID NO.: 38 **AS5-4**: TGTCCACAGTGGAACTTCCA **AS5-7**: TTCCTGGAACATGTTCTCGA SEQ ID NO.: 41

FIG. 9A

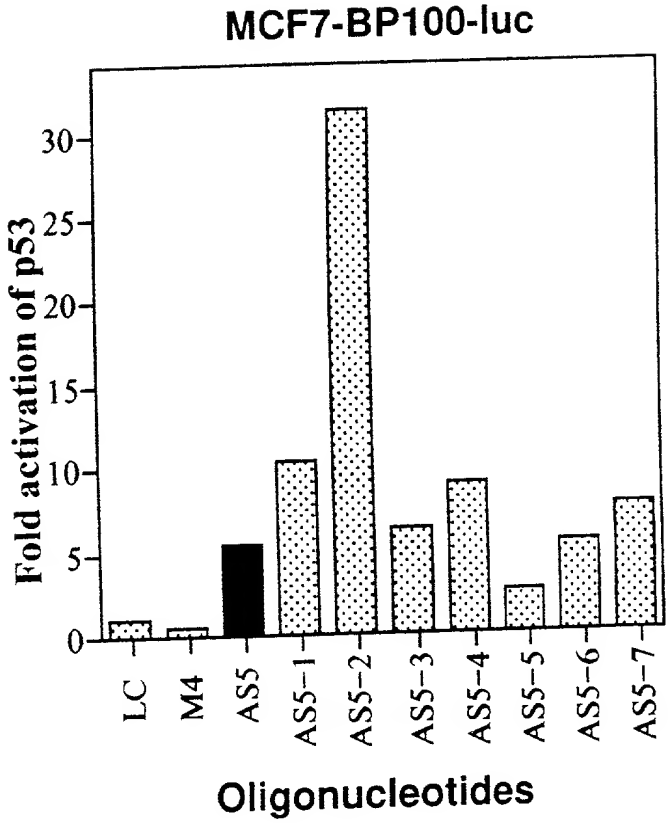

MCF7-BP100-luc
Fold activation of p53
0
5
10
15
20
25
30
LC
M4
AS5
AS5-1
AS5-2
AS5-3
AS5-4
AS5-5
AS5-6
AS5-7
Oligonucleotides

FIG. 9B

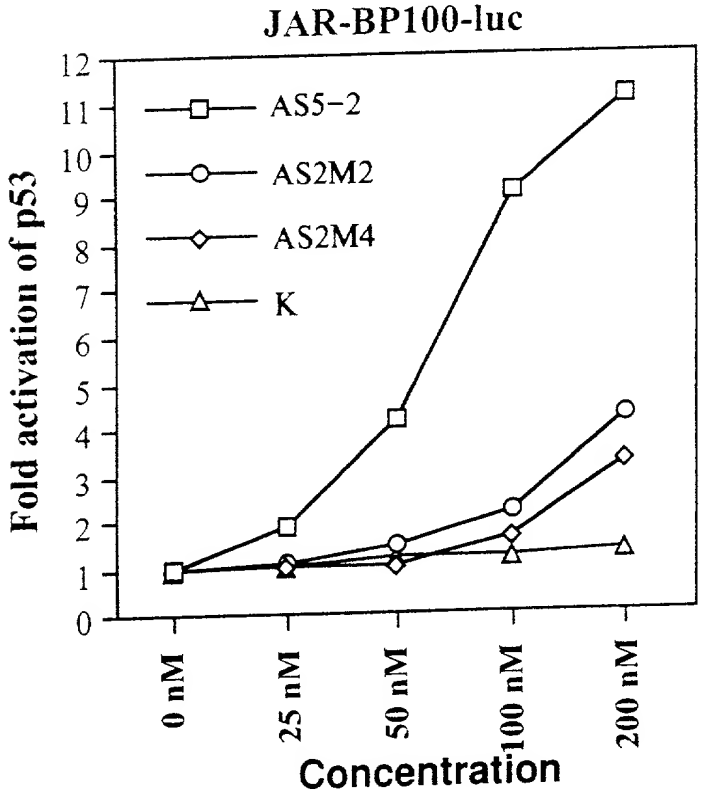

JAR-BP100-luc
Fold activation of p53
0
1
2
3
4
5
6
7
8
9
10
11
12
AS5-2
AS2M2
AS2M4
K
0 nM
25 nM
50 nM
100 nM
200 nM
Concentration

FIG. 9C

FIG. 10A-1

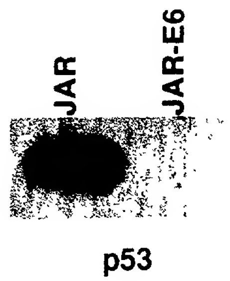


FIG. 10A-2

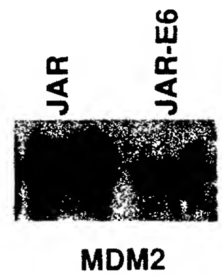


FIG. 10B-1

FIG. 10B-2

FIG. 10B-3

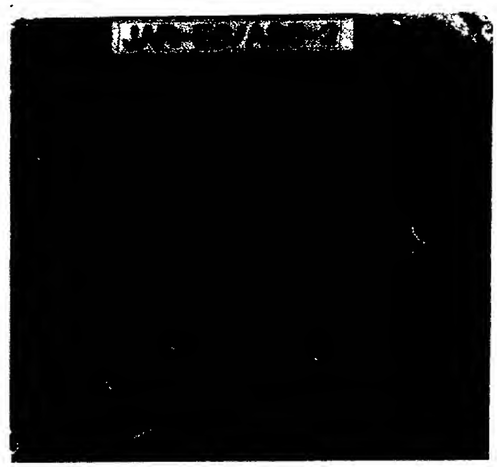


FIG. 10B-4

Control treated

AS5-2 treated

MCF-7
Breast tumor

FIG. 11A

FIG. 11B

SK-N-SH
Neuroblastoma

FIG. 11C

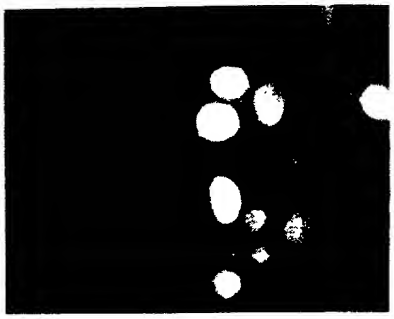


FIG. 11D

A172
Glioblastoma

FIG. 11E

FIG. 11F

HT1080
Fibrosarcoma

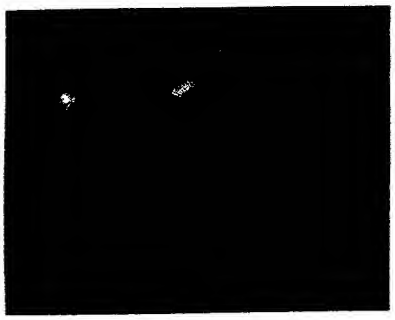


FIG. 11G

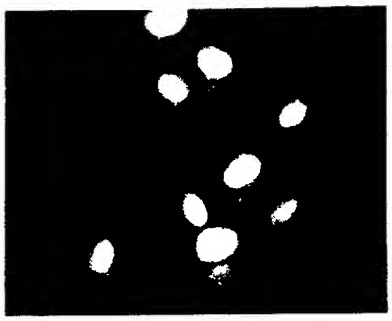


FIG. 11H

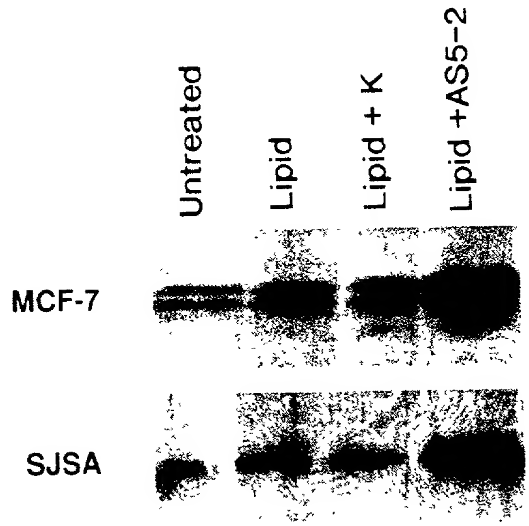


FIG. 12A

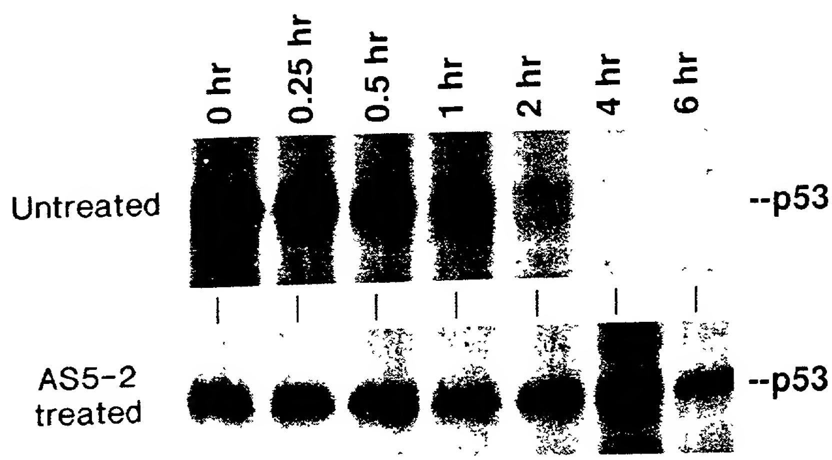


FIG. 12B

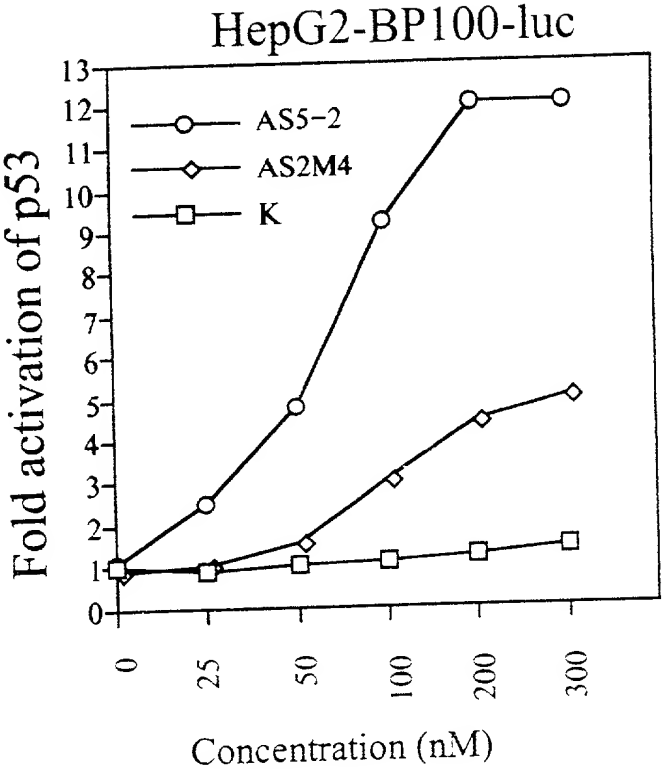


FIG. 13A

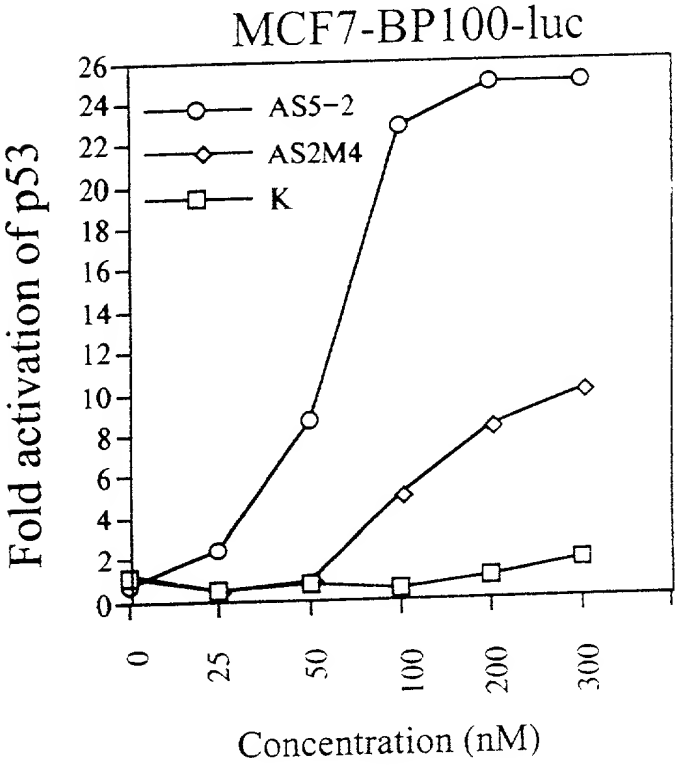


FIG. 13B

FIG. 14A

Control treated

FIG. 14B

AS5-2 treated

HT1080

PA-1

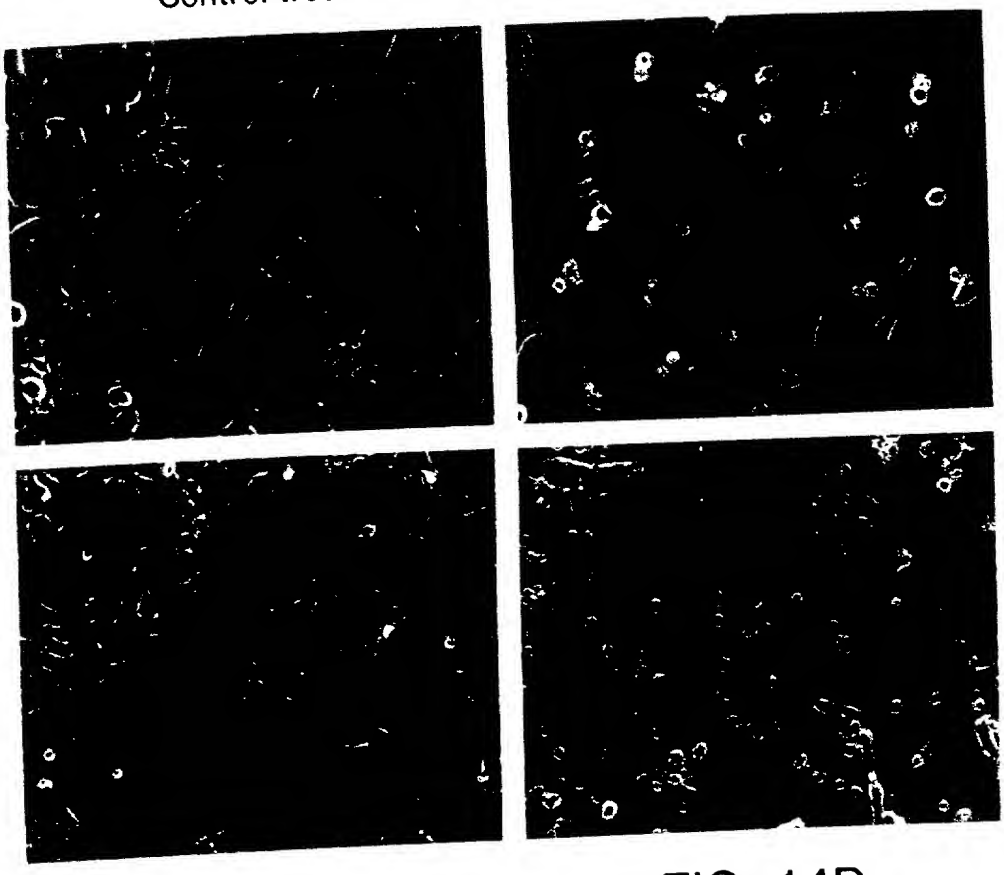


FIG. 14C

FIG. 14D

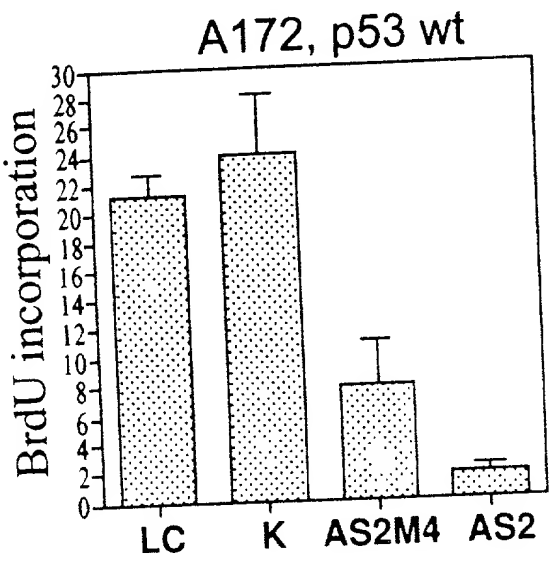


FIG. 15A

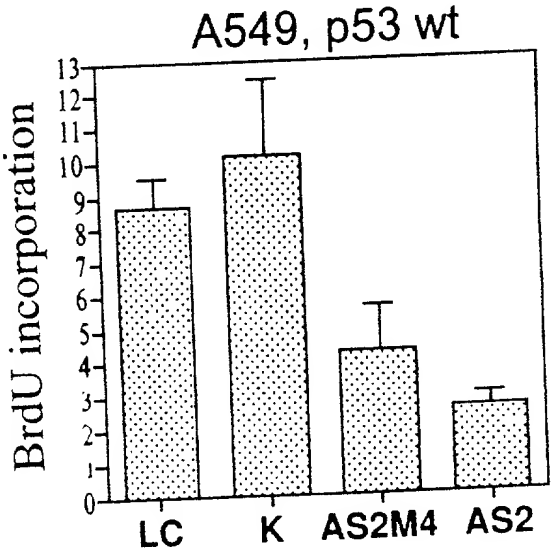


FIG. 15B

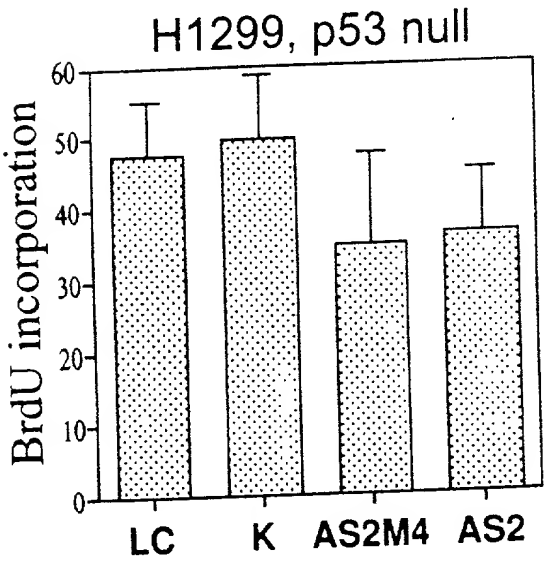


FIG. 15C

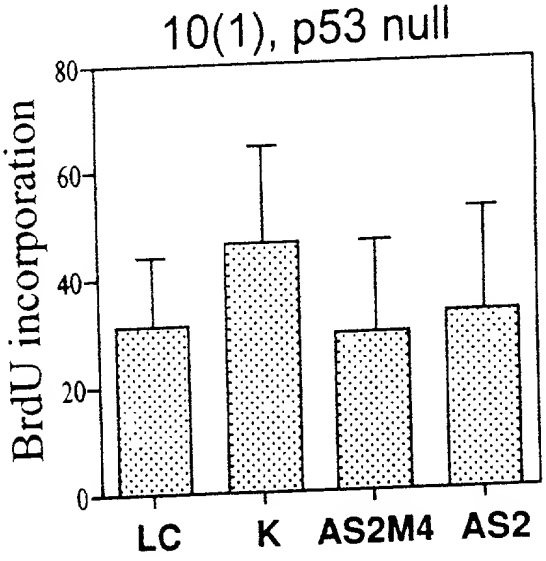


FIG. 15D

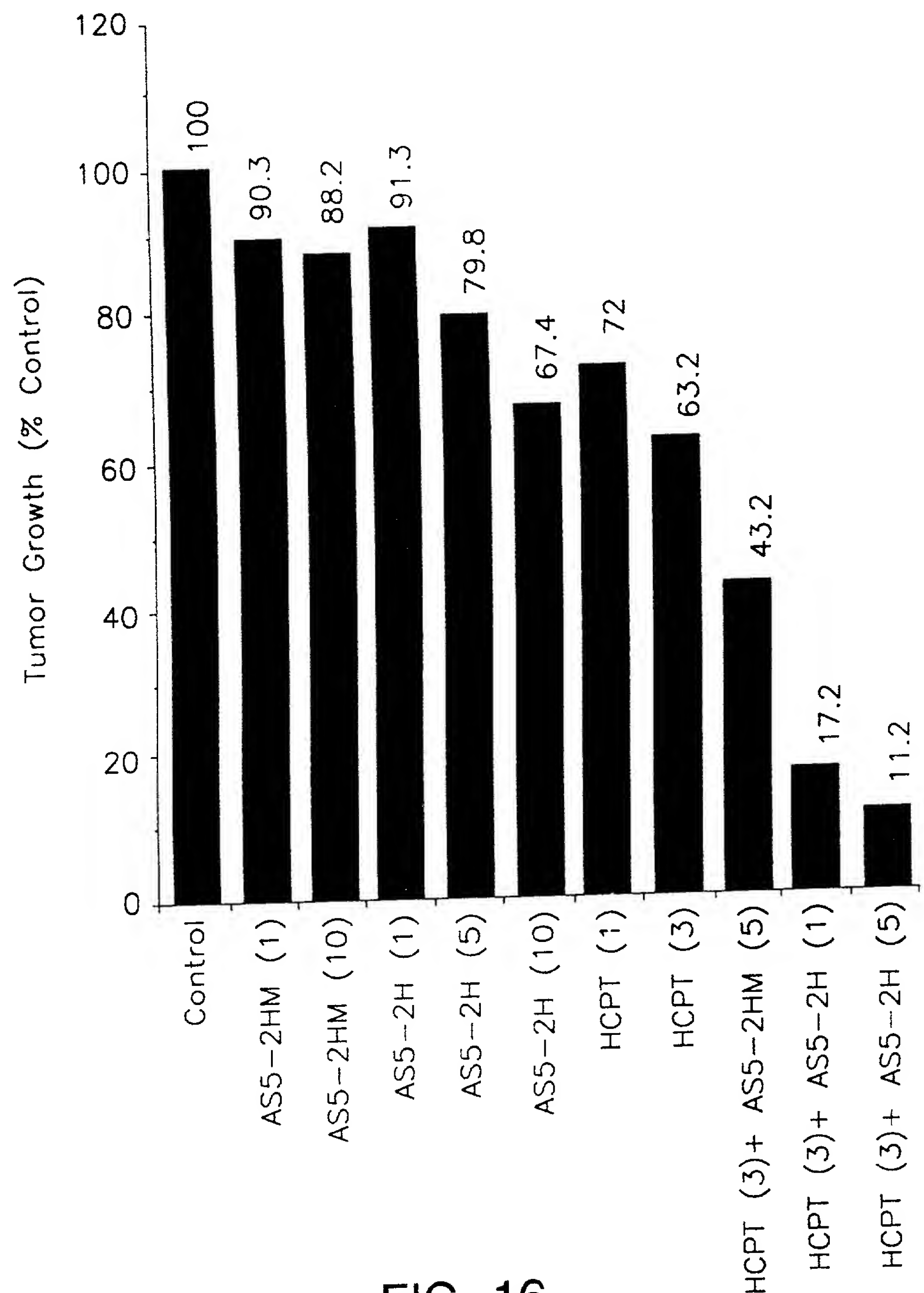

Tumor Growth (% Control)
0
20
40
60
80
100
120
100
90.3
88.2
91.3
79.8
67.4
72
63.2
43.2
17.2
11.2
Control
AS5-2HM (1)
AS5-2HM (10)
AS5-2H (1)
AS5-2H (5)
AS5-2H (10)
HCPT (1)
HCPT (3)
HCPT (3)+ AS5-2HM (5)
HCPT (3)+ AS5-2H (1)
HCPT (3)+ AS5-2H (5)

FIG. 16

Anticancer Activity of Anti-MDM2 Oligonucleotides

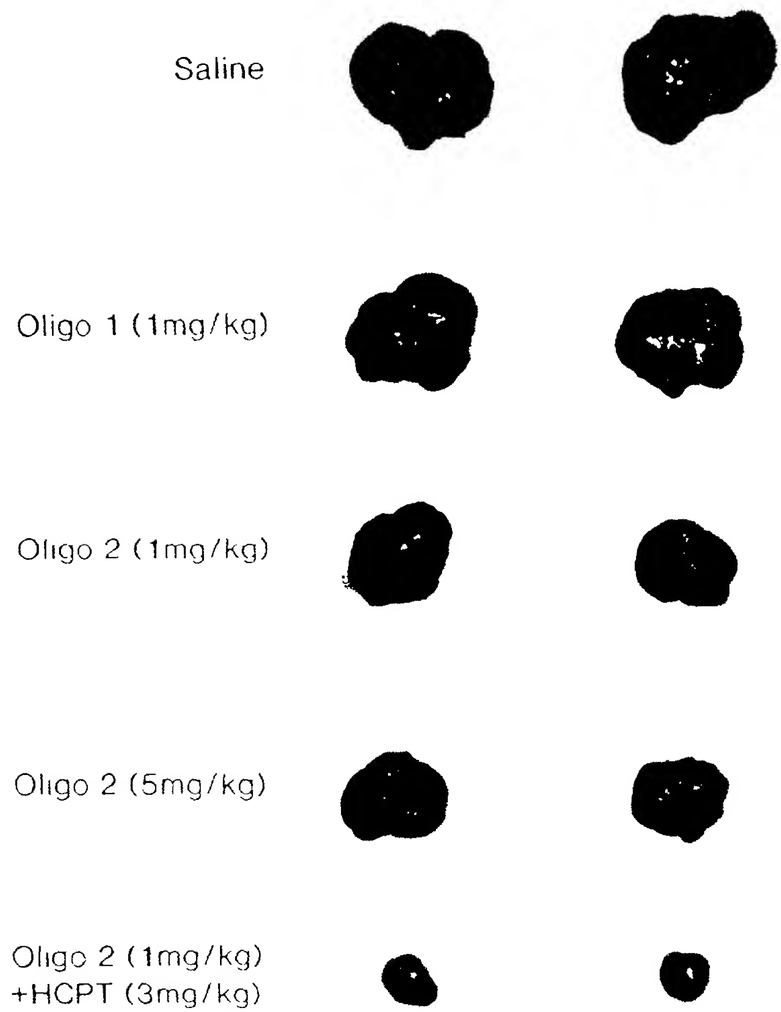


FIG. 17

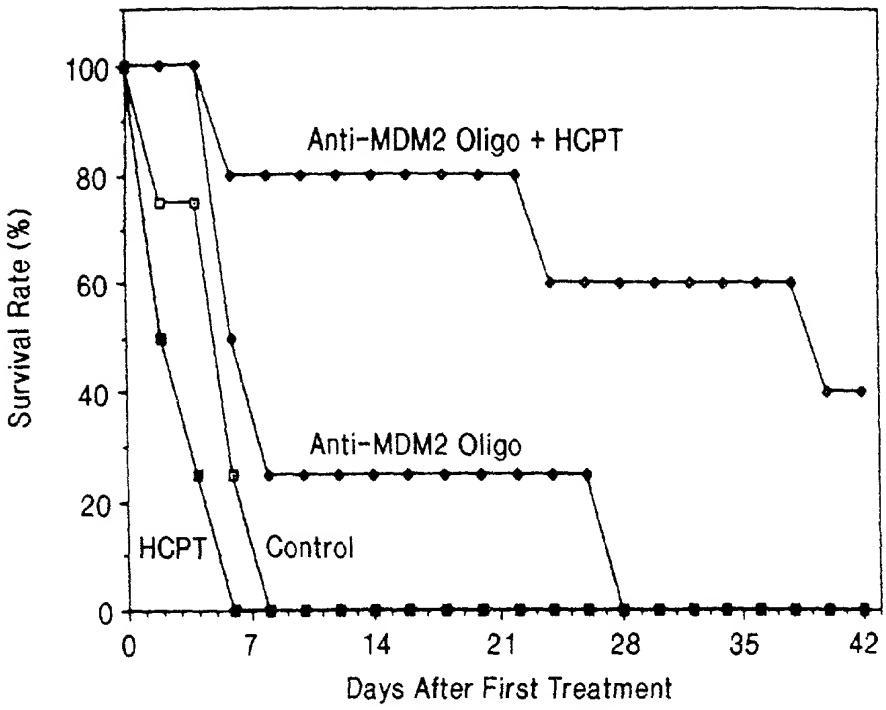


FIG. 18A

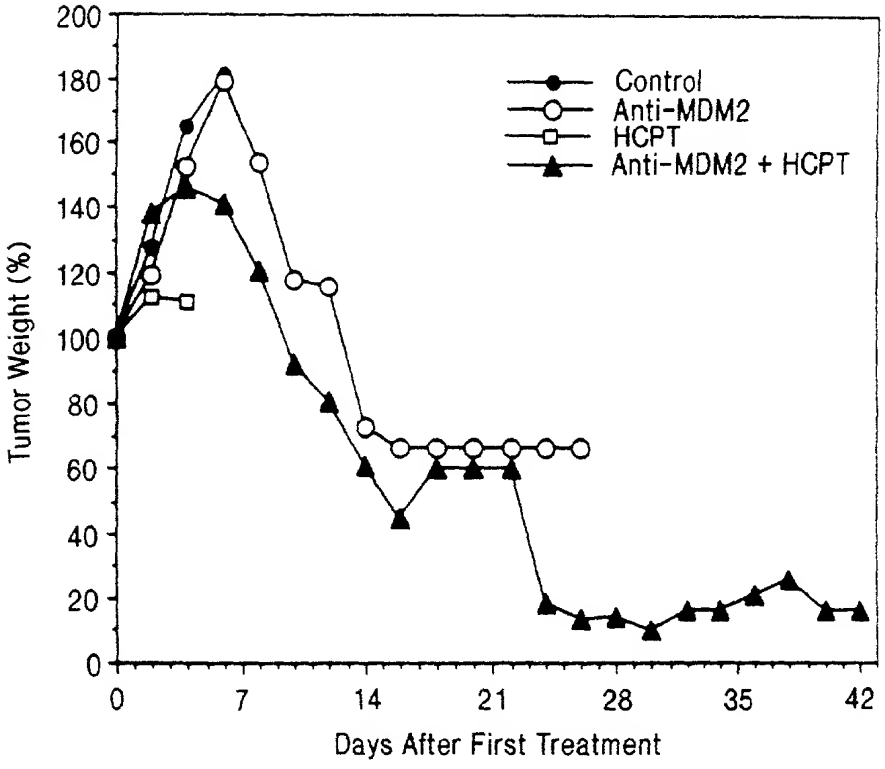


FIG. 18B

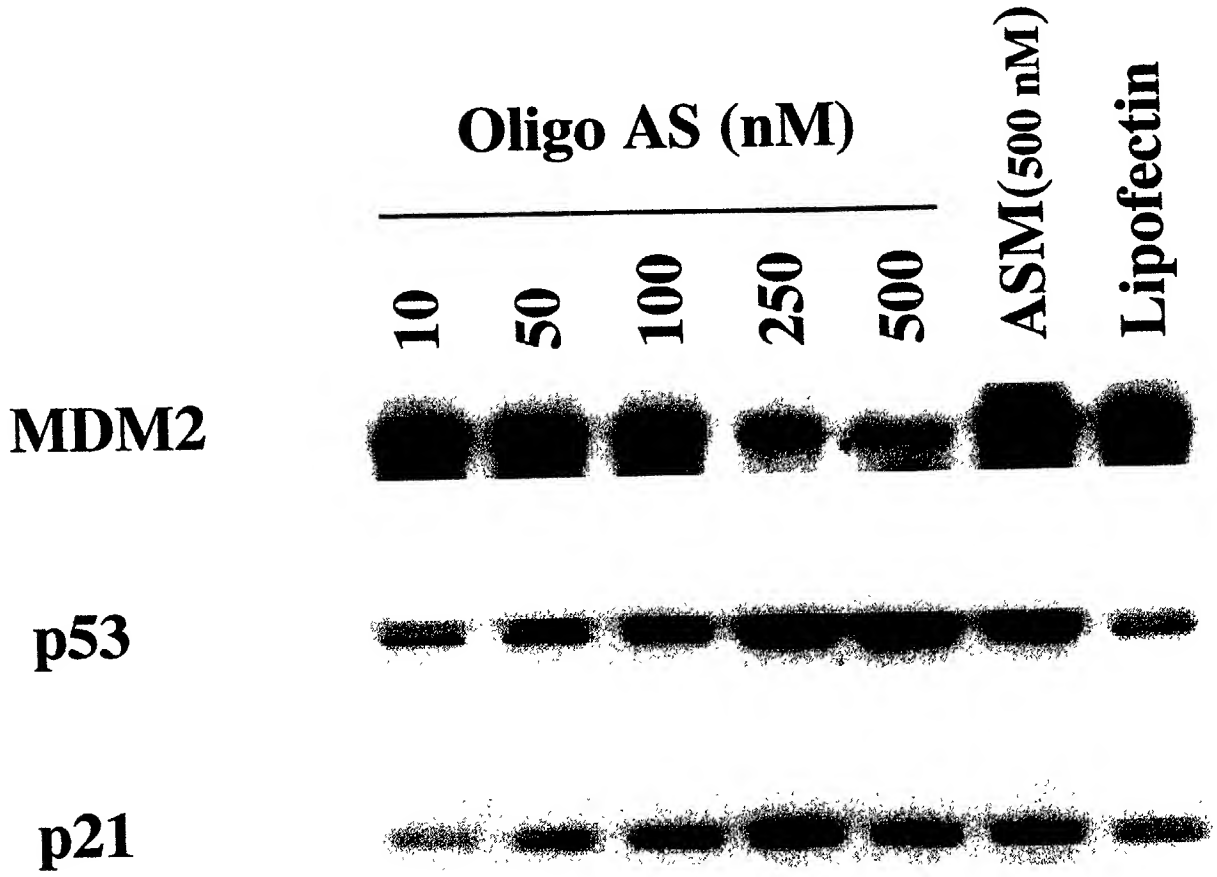

Oligo AS (nM)
10
50
100
250
500
ASM(500 nM)
Lipofectin
MDM2
p53
p21

Fig. 19

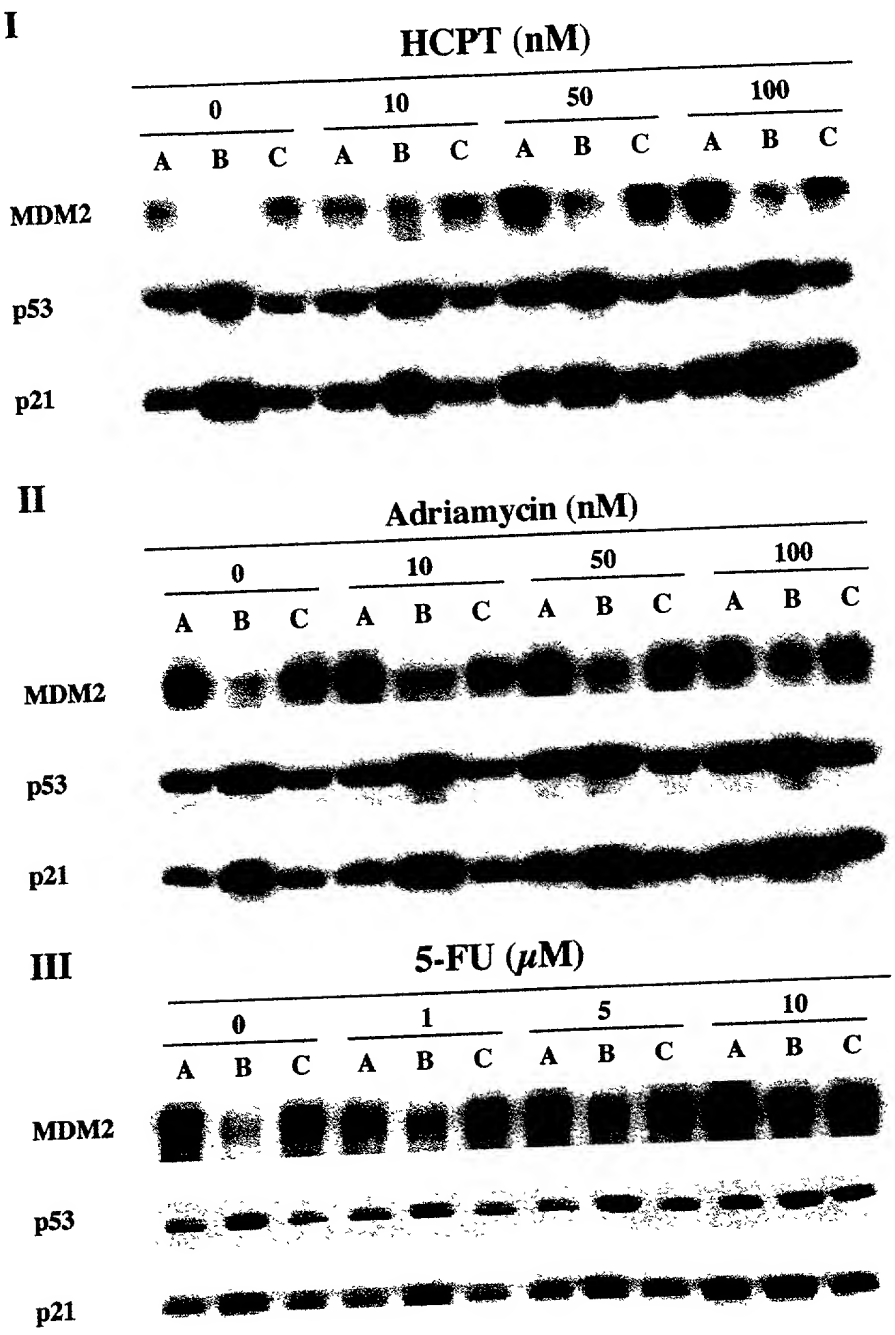

I
HCPT (nM)
0
10
50
100
A B C A B C A B C A B C
MDM2
p53
p21
II
Adriamycin (nM)
0
10
50
100
A B C A B C A B C A B C
MDM2
p53
p21
III
5-FU (μM)
0
1
5
10
A B C A B C A B C A B C
MDM2
p53
p21

Fig. 20

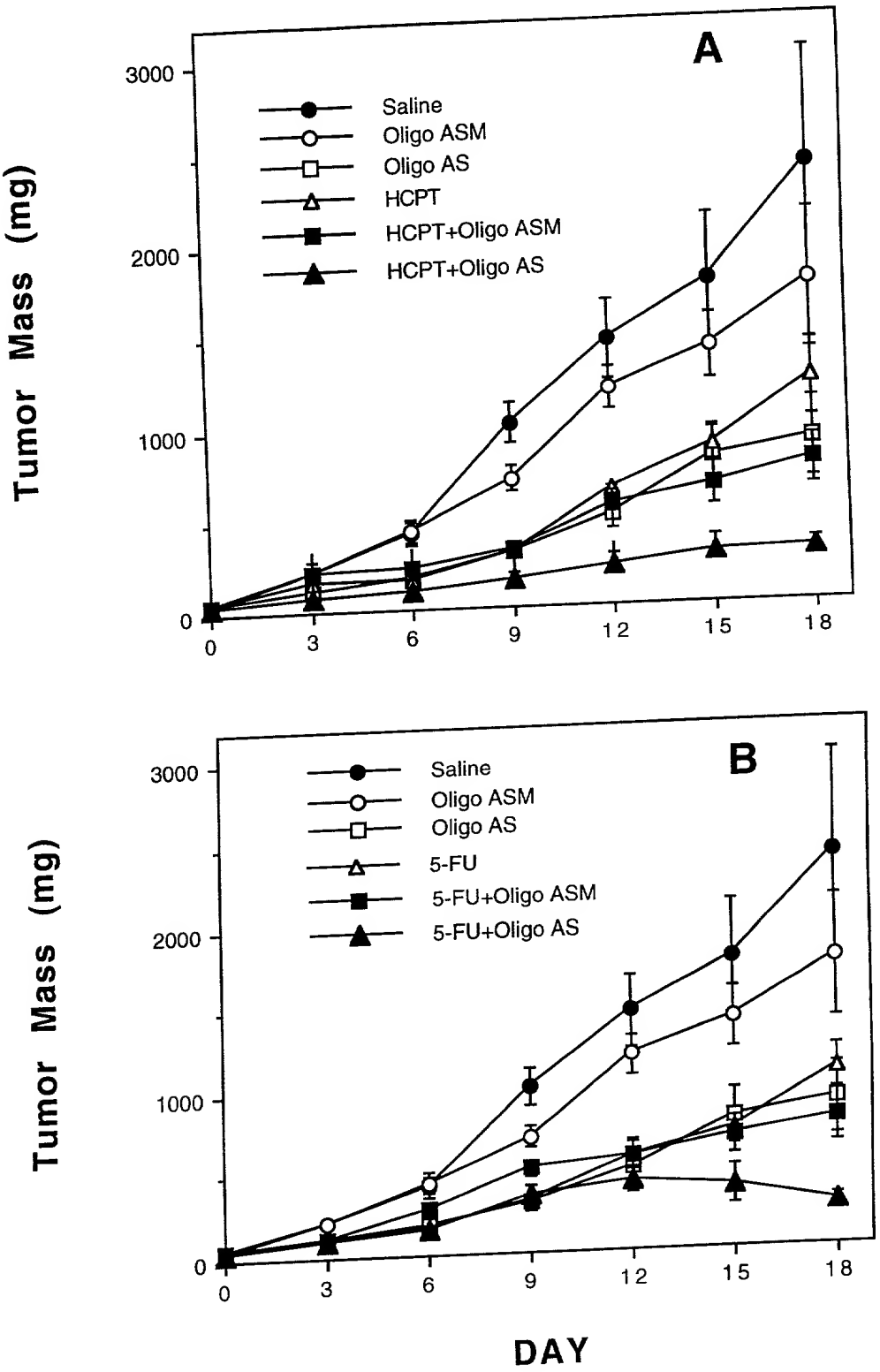
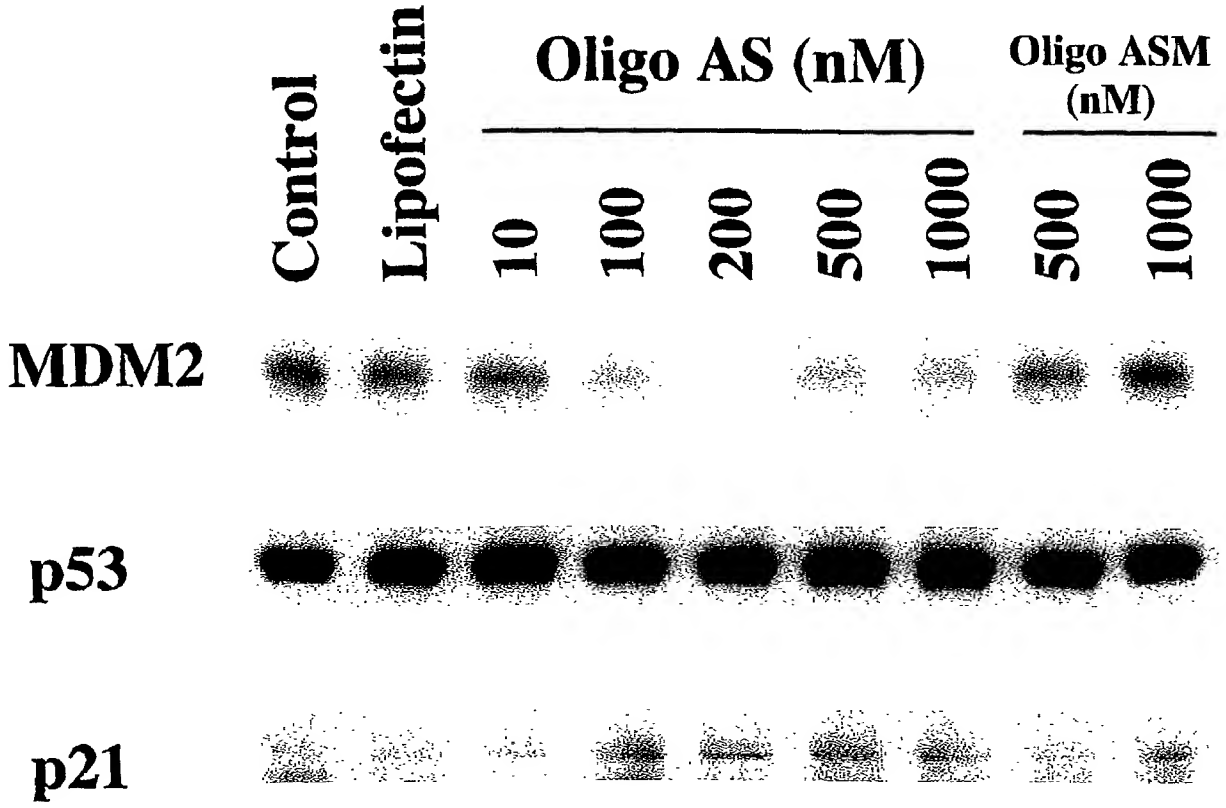

A
Saline
Oligo ASM
Oligo AS
HCPT
HCPT+Oligo ASM
HCPT+Oligo AS
Tumor Mass (mg)
3000
2000
1000
0
0
3
6
9
12
15
18
B
Saline
Oligo ASM
Oligo AS
5-FU
5-FU+Oligo ASM
5-FU+Oligo AS
Tumor Mass (mg)
3000
2000
1000
0
0
3
6
9
12
15
18
DAY

Fig. 21



09541546 .040306

Fig. 22

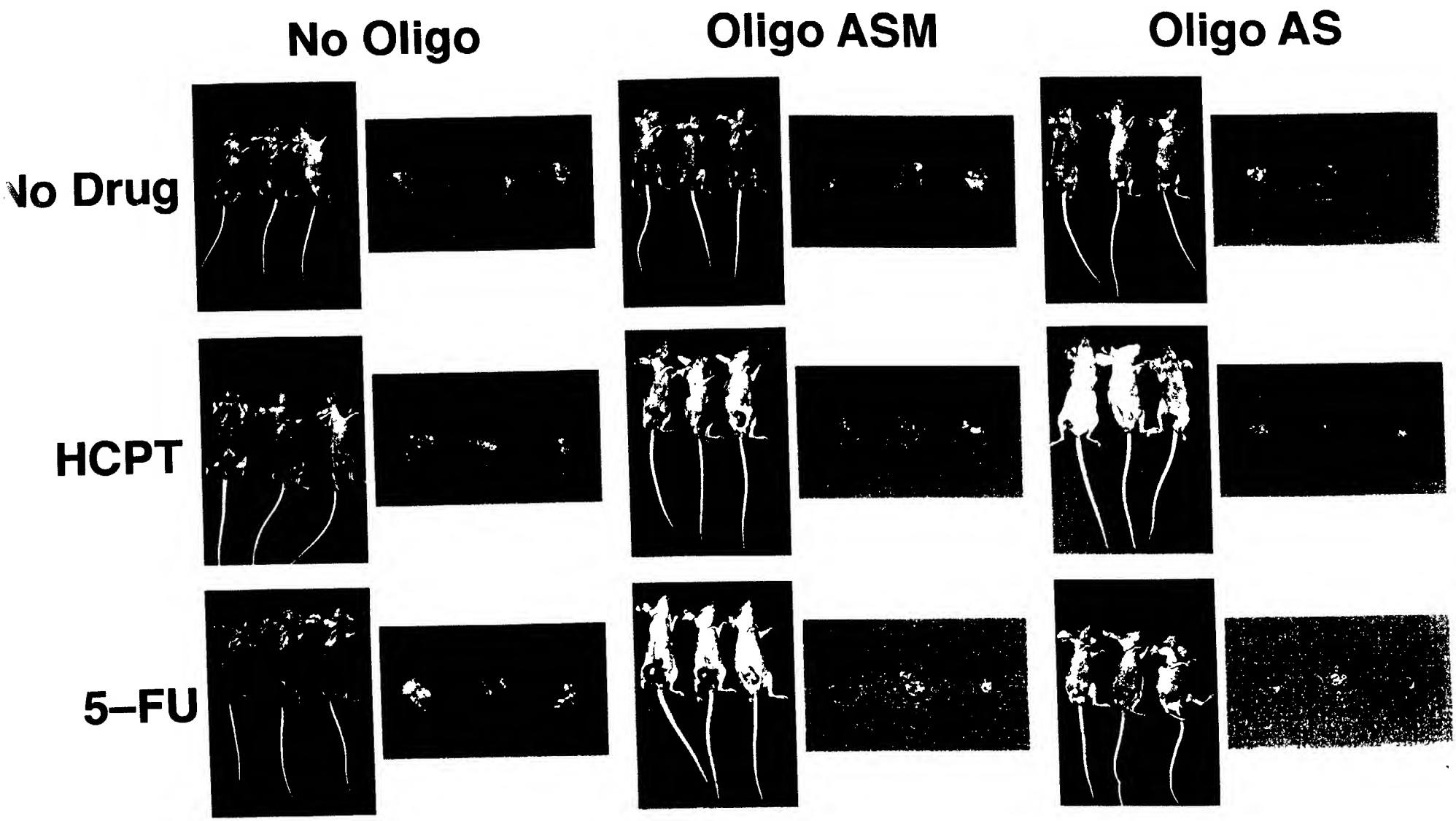


Fig. 23

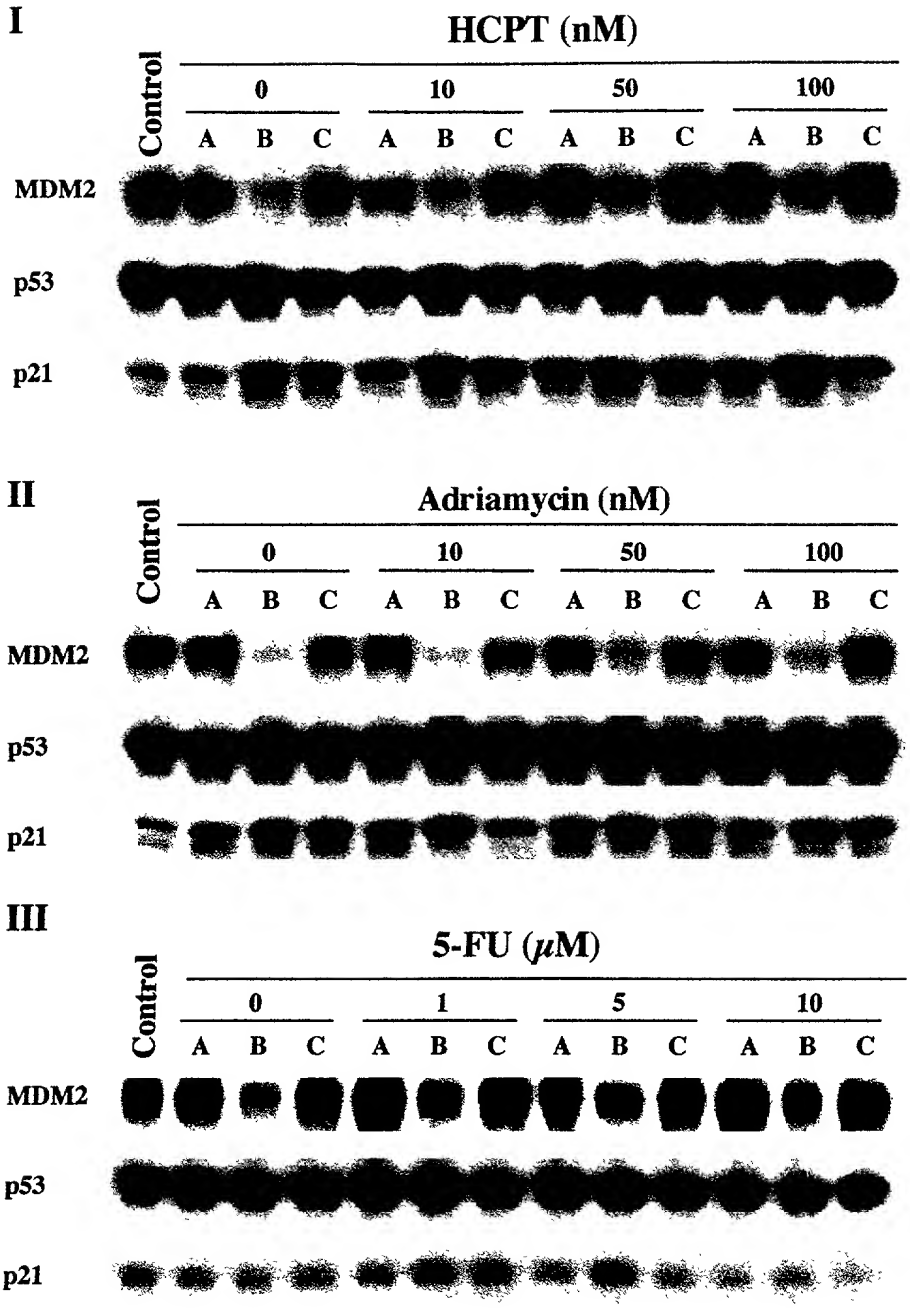

I
HCPT (nM)
Control
0
10
50
100
A B C A B C A B C A B C
MDM2
p53
p21
II
Adriamycin (nM)
Control
0
10
50
100
A B C A B C A B C A B C
MDM2
p53
p21
III
5-FU (μM)
Control
0
1
5
10
A B C A B C A B C A B C
MDM2
p53
p21

Fig. 24

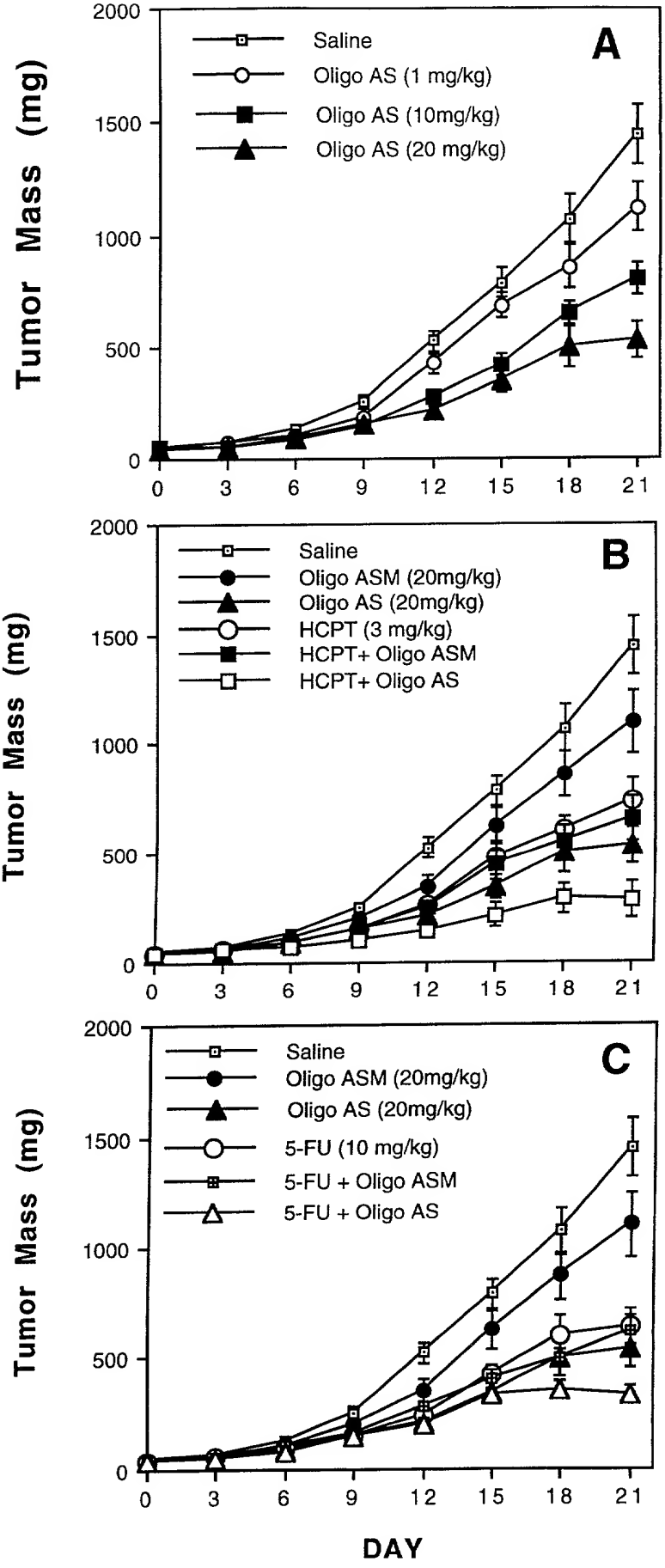

A
Saline
Oligo AS (1 mg/kg)
Oligo AS (10mg/kg)
Oligo AS (20 mg/kg)
Tumor Mass (mg)
2000
1500
1000
500
0
0 3 6 9 12 15 18 21
B
Saline
Oligo ASM (20mg/kg)
Oligo AS (20mg/kg)
HCPT (3 mg/kg)
HCPT+ Oligo ASM
HCPT+ Oligo AS
Tumor Mass (mg)
C
Saline
Oligo ASM (20mg/kg)
Oligo AS (20mg/kg)
5-FU (10 mg/kg)
5-FU + Oligo ASM
5-FU + Oligo AS
Tumor Mass (mg)
DAY

Fig. 25

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Jiandong Chen
Sudhir Agrawal
Ruiwen Zhang

(ii) TITLE OF INVENTION: MDM2-SPECIFIC ANTISENSE OLIGONUCLEOTIDES

(iii) NUMBER OF SEQUENCES: 49

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: McDonnell Boehnen Hulbert & Berghoff
(B) STREET: 300 South Wacker Drive, 32nd Floor
(C) CITY: Chicago
(D) STATE: IL
(E) COUNTRY: United States of America
(F) ZIP: 60606

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Microsoft Word 97

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Greenfield, Michael S.
(B) REGISTRATION NUMBER: 37,147
(C) REFERENCE/DOCKET NUMBER: 98,057-A

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (312) 913-0001
(B) TELEFAX: (312) 913-0002

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2372 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: hmdm2 DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1

```
GCACCGCGCG AGCTTGGCTG CTTCTGGGGC CTGTGTGGCC CTGTGTGTCG GAAAGATGGA   60
GCAAGAAGCC GAGCCCGAGG GGCGGCCGCG ACCCCTCTGA CCGAGATCCT GCTGCTTTCG  120
CAGCCAGGAG CACCGTCCCT CCCCGGATTA GTGCGTACGA GCGCCCAGTG CCCTGGCCCG  180
GAGAGTGGAA TGATCCCCGA GGCCCAGGGC GTCGTGCTTC CGCAGTAGTC AGTCCCCGTG  240
AAGGAAACTG GGGAGTCTTG AGGGACCCCC GACTCCAAGC GCGAAAACCC CGGATGGTGA  300
GGAGCAGGCA AATGTGCAAT ACCAACATGT CTGTACCTAC TGATGGTGCT GTAACCACCT  360
CACAGATTCC AGCTTCGGAA CAAGAGACCC TGGTTAGACC AAAGCCATTG CTTTTGAAGT  420
TATTAAAGTC TGTTGGTGCA CAAAAAGACA CTTATACTAT GAAAGAGGTT CTTTTTTATC  480
TTGGCCAGTA TATTATGACT AAACGATTAT ATGATGAGAA GCAACAACAT ATTGTATATT  540
GTTCAAATGA TCTTCTAGGA GATTTGTTTG GCGTGCCAAG CTTCTCTGTG AAAGAGCACA  600
GGAAAATATA TACCATGATC TACAGGAACT TGGTAGTAGT CAATCAGCAG GAATCATCGG  660
ACTCAGGTAC ATCTGTGAGT GAGAACAGGT GTCACCTTGA AGGTGGGAGT GATCAAAAGG  720
ACCTTGTACA AGAGCTTCAG GAAGAGAAAC CTTCATCTTC ACATTTGGTT TCTAGACCAT  780
CTACCTCATC TAGAAGGAGA GCAATTAGTG AGACAGAAGA AAATTCAGAT GAATTATCTG  840
GTGAACGACA AAGAAAACGC CACAAATCTG ATAGTATTTC CCTTTCCTTT GATGAAAGCC  900
TGGCTCTGTG TGTAATAAGG GAGATATGTT GTGAAAGAAG CAGTAGCAGT GAATCTACAG  960
GGACGCCATC GAATCCGGAT CTTGATGCTG GTGTAAGTGA ACATTCAGGT GATTGGTTGG 1020
ATCAGGATTC AGTTTCAGAT CAGTTTAGTG TAGAATTTGA AGTTGAATCT CTCGACTCAG 1080
AAGATTATAG CCTTAGTGAA GAAGGACAAG AACTCTCAGA TGAAGATGAT GAGGTATATC 1140
AAGTTACTGT GTATCAGGCA GGGGAGAGTG ATACAGATTC ATTTGAAGAA GATCCTGAAA 1200
TTTCCTTAGC TGACTATTGG AAATGCACTT CATGCAATGA AATGAATCCC CCCCTTCCAT 1260
CACATTGCAA CAGATGTTGG GCCCTTCGTG AGAATTGGCT TCCTGAAGAT AAAGGGAAAG 1320
ATAAAGGGGA AATCTCTGAG AAAGCCAAAC TGGAAAACTC AACACAAGCT GAAGAGGGCT 1380
TTGATGTTCC TGATTGTAAA AAAACTATAG TGAATGATTC CAGAGAGTCA TGTGTTGAGG 1440
AAAATGATGA TAAAATTACA CAAGCTTCAC AATCACAAGA AAGTGAAGAC TATTCTCAGC 1500
CATCAACTTC TAGTAGCATT ATTTATAGCA GCCAAGAAGA TGTGAAAGAG TTTGAAAGGG 1560
AAGAAACCCA AGACAAAGAA GAGAGTGTGG AATCTAGTTT GCCCCTTAAT GCCATTGAAC 1620
CTTGTGTGAT TTGTCAAGGT CGACCTAAAA ATGGTTGCAT TGTCCATGGC AAAACAGGAC 1680
ATCTTATGGC CTGCTTTACA TGTGCAAAGA AGCTAAAGAA AAGGAATAAG CCCTGCCCAG 1740
TATGTAGACA ACCAATTCAA ATGATTGTGC TAACTTATTT CCCCTAGTTG ACCTGTCTAT 1800
AAGAGAATTA TATATTTCTA ACTATATAAC CCTAGGAATT TAGACAACCT GAAATTTATT 1860
CACATATATC AAAGTGAGAA AATGCCTCAA TTCACATAGA TTTCTTCTCT TTAGTATAAT 1920
TGACCTACTT TGGTAGTGGA ATAGTGAATA CTTACTATAA TTTGACTTGA ATATGTAGCT 1980
CATCCTTTAC ACCAACTCCT AATTTTAAAT AATTTCTACT CTGTCTTAAA TGAGAAGTAC 2040
TTGGTTTTTT TTTTCTTAAA TATGTATATG ACATTTAAAT GTAACTTATT ATTTTTTTTG 2100
AGACCGAGTC TTGCTCTGTT ACCCAGGCTG GAGTGCAGTG GGTGATCTTG GCTCACTGCA 2160
AGCTCTGCCC TCCCCGGGTT CGCACCATTC TCCTGCCTCA GCCTCCCAAT TAGCTTGGCC 2220
TACAGTCATC TGCCACCACA CCTGGCTAAT TTTTTGTACT TTTAGTAGAG ACAGGGTTTC 2280
ACCGTGTTAG CCAGGATGGT CTCGATCTCC TGACCTCGTG ATCCGCCCAC CTCGGCCTCC 2340
CAAAGTGCTG GGATTACAGG CATGAGCCAC CG                  2372
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2

TTGGCCAGTA TATTATGACT 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3

CCTTGAAGGT GGGAGTGATC 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4

TGGATCAGGA TTCAGTTTCA 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5

GATGACTCAC ACCATCATGG 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6

CAGAGCCTTC ATCTTCCCAG 20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7

ACCTCACAGA TTCCAGCTTC 20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8

CCAGCTTCGG AACAAGAGAC 20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9

TCTACCTCAT CTAGAAGGAG 20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10

TCCTTAGCTG ACTATTGGAA 20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11

TCATGCAATG AAATGAATCC 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1710 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12

GAGGAGCCGC CGCCTTCTCG TCGCTCGAGC TCTGGACGAC CATGGTCGCT CAGGCCCCGT 60
CCGCGGGGCC TCCGCGCTCC CCGTGAAGGG TCGGAAGATG CGCGGGAAGT AGCAGCCGTC 120
TGCTGGGCGA GCGGGAGACC GACCGGACAC CCCTGGGGGA CCCTCTCGGA TCACCGCGCT 180
TCTCCTGCGG CCTCCAGGCC AATGTGCAAT ACCAACATGT CTGTGTCTAC CGAGGGTGCT 240
GCAAGCACCT CACAGATTCC AGCTTCGGAA CAAGAGACTC TGGTTAGACC AAAACCATTG 300
CTTTTGAAGT TGTTAAAGTC CGTTGGAGCG CAAAACGACA CTTACACTAT GAAAGAGATT 360
ATATTTTATA TTGGCCAGTA TATTATGACT AAGAGGTTAT ATGACGAGAA GCAGCAGCAC 420
ATTGTGTATT GTTCAAATGA TCTCCTAGGA GATGTGTTTG GAGTCCCGAG TTTCTCTGTG 480
AAGGAGCACA GGAAAATATA TGCAATGATC TACAGAAATT TAGTGGCTGT AAGTCAGCAA 540

GACTCTGGCA CATCGCTGAG TGAGAGCAGA CGTCAGCCTG AAGGTGGGAG TGATCTGAAG 600
GATCCTTTGC AAGCGCCACC AGAAGAGAAA CCTTCATCTT CTGATTTAAT TTCTAGACTG 660
TCTACCTCAT CTAGAAGGAG ATCCATTAGT GAGACAGAAG AGAACACAGA TGAGCTACCT 720
GGGGAGCGGC ACCGGAAGCG CCGCAGGTCC CTGTCCTTTG ATCCGAGCCT GGGTCTGTGT 780
GAGCTGAGGG AGATGTGCAG CGGCGGCACG AGCAGCAGTA GCAGCAGCAG CAGCGAGTCC 840
ACAGAGACGC CCTCGCATCA GGATCTTGAC GATGGCGTAA GTGAGCATTC TGGTGATTGC 900
CTGGATCAGG ATTCAGTTTC TGATCAGTTT AGCGTGGAAT TTGAAGTTGA GTCTCTGGAC 960
TCGGAAGATT ACAGCCTGAG TGACGAAGGG CACGAGCTCT CAGATGAGGA TGATGAGGTC 1020
TATCGGGTCA CAGTCTATCA GACAGGAGAA AGCGATACAG ACTCTTTTGA AGGAGATCCT 1080
GAGATTTCCT TAGCTGACTA TTGGAAGTGT ACCTCATGCA ATGAAATGAA TCCTCCCCTT 1140
CCATCACACT GCAAAAGATG CTGGACCCTT CGTGAGAACT GGCTTCCAGA CGATAAGGGG 1200
AAAGATAAAG TGGAAATCTC TGAAAAAGCC AAACTGGAAA ACTCAGCTCA GGCAGAAGAA 1260
GGCTTGGATG TGCCTGATGG CAAAAAGCTG ACAGAGAATG ATGCTAAAGA GCCATGTGCT 1320
GAGGAGGACA GCGAGGAGAA GGCCGAACAG ACGCCCCTGT CCCAGGAGAG TGACGACTAT 1380
TCCCAACCAT CGACTTCCAG CAGCATTGTT TATAGCAGCC AAGAAAGCGT GAAAGAGTTG 1440
AAGGAGGAAA CGCAGCACAA AGACGAGAGT GTGGAATCTA GCTTCTCCCT GAATGCCATC 1500
GAACCATGTG TGATCTGCCA GGGGCGGCCT AAAAATGGCT GCATTGTTCA CGGCAAGACT 1560
GGACACCTCA TGTCATGTTT CACGTGTGCA AAGAAGCTAA AAAAAAGAAA CAAGCCCTGC 1620
CCAGTGTGCA GACAGCCAAT CCAAATGATT GTGCTAAGTT ACTTCAACTA GCTGACCTGC 1680
TCACAAAAAT AGAATTTTAT ATTTCTAACT 1710

(2) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13

ACATCTGTGA GTGAGAACAG 20

(2) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14

GTGAGTGAGA ACAGGTGTCA 20

(2) INFORMATION FOR SEQ ID NO:15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15

TGAGAACAGG TGTCACCTTG 20

(2) INFORMATION FOR SEQ ID NO:16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16

ACAGGTGTCA CCTTGAAGGT 20

(2) INFORMATION FOR SEQ ID NO:17:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17

TGGGAGTGAT CAAAAGGACC 20

(2) INFORMATION FOR SEQ ID NO:18:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18

GTGATCAAAA GGACCTTGTA 20

(2) INFORMATION FOR SEQ ID NO:19:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19

AAGGACCTTG TACAAGAGCT 20

(2) INFORMATION FOR SEQ ID NO:20:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20

TGAACATTCA GGTGATTGGT 20

(2) INFORMATION FOR SEQ ID NO:21:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21

ATTCAGGTGA TTGGTTGGAT 20

(2) INFORMATION FOR SEQ ID NO:22:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22

AGGTGATTGG TTGGATCAGG A 21

(2) INFORMATION FOR SEQ ID NO:23:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23

ATTCAGTTTC AGATCAGTTT 20

(2) INFORMATION FOR SEQ ID NO:24:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24

GATCAGTTTA GTGTAGAATT 20

(2) INFORMATION FOR SEQ ID NO:25:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25

TGACACTTGT TCTTACTCAC 20

(2) INFORMATION FOR SEQ ID NO:26:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26

TGACTCTTGT CCTTACTCAC 20

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27

AGTCATAATA TACTGGCCAA 20

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28

GATCACTCCC ACCTTCAAGG 20

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29

TGAAACTGAA TCCTGATCCA 20

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30

GAAGCTGGAA TCTGTGAGGT 20

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31

GTCTCTTGTT CCGAAGCTGG 20

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32

CTCCTTCTAG ATGAGGTAGA 20

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33

TTCCAATAGT CAGCTAAGGA 20

(2) INFORMATION FOR SEQ ID NO:34:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34

GGATTCATTT CATTGCATGA 20

(2) INFORMATION FOR SEQ ID NO:35:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35

CTGTTCTCAC TCACAGATGT 20

(2) INFORMATION FOR SEQ ID NO:36:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36

TGACACCTGT TCTCACTCAC 20

(2) INFORMATION FOR SEQ ID NO:37:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37

CAAGGTGACA CCTGTTCTCA 20

(2) INFORMATION FOR SEQ ID NO:38:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38

ACCTTCAAGG TGACACCTGT 20

(2) INFORMATION FOR SEQ ID NO:39:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39

GGTCCTTTTG ATCACTCCCA 20

(2) INFORMATION FOR SEQ ID NO:40:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40

TACAAGGTCC TTTTGATCAC 20

(2) INFORMATION FOR SEQ ID NO:41:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41

AGCTCTTGTA CAAGGTCCTT 20

(2) INFORMATION FOR SEQ ID NO:42:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42

ACCAATCACC TGAATGTTCA 20

(2) INFORMATION FOR SEQ ID NO:43:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43

ATCCAACCAA TCACCTGAAT 20

(2) INFORMATION FOR SEQ ID NO:44:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44

TCCTGATCCA ACCAATCACC T 21

(2) INFORMATION FOR SEQ ID NO:45:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45

AAACTGATCT GAAACTGAAT 20

(2) INFORMATION FOR SEQ ID NO:46:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46

AATTCTACAC TAAACTGATC 20

(2) INFORMATION FOR SEQ ID NO:47:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47

UGACACCTGT TCTCACUCAC 20

(2) INFORMATION FOR SEQ ID NO:48:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48

UGAGACCAGT TGTCAGUCAC 20

(2) INFORMATION FOR SEQ ID NO:49:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49

AAGCTCTTGT ACAAGGTCCT TTTGATCACT CCCACCTTCA AGGTGACACC TGTTCTCACT 60
CACAGATGTA CCT 73